Roles of OsCCD1 in Carotenoid Catabolism in Rice Seeds

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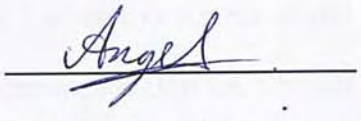
Statement

All the experiments reported in this thesis were performed by the author, unless specially stated otherwise in the content.



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Abstract

Vitamin A is an essential nutrient for human health. Vitamin A Deficiency (VAD) causes weakened immune system, night blindness, or even complete blindness. Currently, over one hundred million people are suffering from VAD, of which about 250 to 300 thousands children become blind each year. To combat this problem, one of the promising ways is to increase pro-vitamin A accumulation in staple food. This can be done by reconstructing the carotenoid biosynthetic pathway in plants via genetic engineering. Examples have been demonstrated in rice, wheat, maize and potato.

While much of the effort has been focused on carotenoid biosynthesis, potential catabolism received little attention. Carotenoid cleavage dioxygenases (CCDs) have recently been implicated in the catabolism of carotenoids. Here, we studied the role of OsCCDs in the catabolism of carotenoids in developing rice seeds. RT-PCR analyses indicated that carotenoid biosynthetic genes were expressed in developing rice seeds, but *PSY1* expression is significantly low. Analysis of the rice genome identified nine CCDs, of which three (OsNCED3, OsNCED5 and OsNCED9) are believed to be involved in the synthesis of ABA, three (OsCCD7, OsCCD8a and OsCCD8b) involved in the synthesis of strigolactone, three (OsCCD1, OsCCD4a and OsCCD4b) are involved in the production of aromatic volatiles. RT-PCR analysis indicated that only *OsCCD1* is highly expressed in the developing rice seeds. Using *in vivo* enzyme activity assay in bacteria, we demonstrated that OsCCD1 protein is enzymatically active in cleaving a broad spectrum of carotenoids, implying that OsCCD1 is capable of degrading

carotenoids in rice seeds. To analyze this degradation genetically, we created transgenic rice with knockdown expression of *OsCCD1* driven by an endosperm specific promoter and a constitutive promoter respectively. T_1 transgenic seeds showed distinct yellowing in pigmentation, suggesting accumulation of carotenoids although in a small amount. To determine the role of OsCCD1 in seeds with high carotenoids, we created transgenic lines over-expressing *CRTI/ZmPSY1*, the biosynthetic pathway in the Golden rice 2, in conjunction with *OsCCD1* RNAi. The seeds displayed much intense yellowing, indicating a dramatic increase in carotenoid accumulation in rice seeds. Taken together, these results indicate that 1) an active carotenoid biosynthetic pathway exits in rice endosperm but only synthesizes a limited amount of carotenoids due to low *PSY1* expression; 2) OsCCD1 plays a dominant role in carotenoid catabolism in rice seeds and attenuation of its expression in conjunction with enhanced biosynthesis may provide an elevated accumulation in carotenoids in rice seeds.

摘要

維生素 A 是人體必需的營養之一。 缺乏维生素 A 會引起以眼和皮膚病變爲主的全身性疾病。目前，世界上有超過一億人患有維生素 A 缺乏症, 其中每年約有二十至三十萬的兒童甚至因此失明。爲了有效地解決這個問題，科學界正研究通過轉基因的方法，重建類胡蘿蔔素(carotenoids)在植物體內的合成途徑，從而增加人類主要食糧中維生素原 A (pro-vitamin A)的合成。目前，此方法已成功的應用在水稻，小麥，玉米和馬鈴薯等主要農作物上。

近年，由於類胡蘿蔔素裂解雙加氧酶 (Carotenoid cleavage dioxygenases; CCD) 的發現，類胡蘿蔔素的代謝作用始爲人所重視。CCD 最廣爲人知的作用，是其在獨角金內酯 (strigolactone) 和脫落酸 (abscisic acid) 的合成作用中扮演着重要的角色。而在水稻種子發育的過程中，我們對於 CCD 的作用仍所知甚少。本次研究發現 CCD 基因家族在水稻基因組中有九個成員，其中三個（OsNCED3，OsNCED5 和 OsNCED9）被認爲參與了脫落酸的合成，三個（OsCCD7，OsCCD8a 和 OsCCD8b）被認爲參與了獨角金內酯的合成，而其餘三個(OsCCD1, OsCCD4a 和 OsCCD4b)則被認爲參與了揮發性香味的合成 。根據逆轉錄聚合酶鏈式反應(RT-PCR) 的分析，只有 OsCCD1 在水稻種子中高度表達。在細菌體內，OsCCD1 對不同類型的類胡蘿蔔素底物均有裂解作用。這意味着 OsCCD1 可能對水稻種子中類胡蘿蔔素的降解有着重大作用。爲了進一步研究，我們從水稻中克隆了 *OsCCD1* 的互補脫氧核糖核酸(cDNA)，並借助组成型表达啓動子和種子特異性啓動子的驅動，分別在所有組織和胚乳中抑制了 *OsCCD1* 基因的表達。與野生水稻種子相比，轉基因 T_1 種子有輕微的顏色轉變，證明其類胡蘿蔔素的含量只有很少的變化。這結果

正合乎類胡蘿蔔素在水稻種子中並非沒有，而是只有有限度合成的推論。爲進一步提高類胡蘿蔔素的含量，我們在水稻胚乳中表達了兩個曾用於構建黃金水稻的維生素原 A 生成酶，即八氫番茄紅素合成酶（PSY）和八氫番茄紅素脫氫酶 (CRTI) ，並同時抑制 *OsCCD1* 的表達。結果轉基因種子明顯轉黃，證明類胡蘿蔔素的含量有着顯著的提高。

通過本次研究, 我們得出以下兩點結論。第一，雖然八氫番茄紅素合成酶的表達量很低，但在水稻種子中類胡蘿蔔素的合成途徑是確實存在，並且是活躍的。第二，在水稻種子中，OsCCD1 擔當着分解代謝類胡蘿蔔素的主要角色。因此，在增加類胡蘿蔔素合成時，同時抑制 *OsCCD1* 的表達，能夠再進一步提高類胡蘿蔔素(即維生素原 A) 在水稻種子中的含量。

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List of Abbreviations

BCMO	β,β'-carotene 15, 15'-monooxygenase
CCD	Carotenoid cleavage dioxygenase
cDNA	Complementary deoxyribonucleic acid
DAF	Day(s) after flowering
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dCTP	Deoxynucleotide
dGTP	Deoxyguanine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymine triphosphate
E.coli	*Escherichia coli*
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization of the United Nations
GST	Glutathione S-transferase
GUS	β-Glucuronidase reporter gene
HPLC	High pressure liquid chromatography
LB	Luria Broth
NCED	Nine-cis-epoxycarotenoid dioxygenase
NEB	New England BioLabs, Inc
OD_{600}	Optical density measured at 600nm
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
WHO	The World Health Organization

Chapter 1. General introduction

Rice is the staple food for more than half of the world population. Although rich in carbohydrates and dietary fiber, rice is extremely low in pro-vitamin A carotenoids. Vitamin A is an essential nutrient for the development of eye and immune system. Deficiency of vitamin A can causes night blindness or even complete blindness, weakened immune system, increased susceptibility to certain cancers, heart diseases and age-related macular degeneration (Cooper et al., 1999; Landrum and Bone, 2004).

As humans and animals cannot synthesize vitamin A, it must be obtained from diet, either in form of retinol from animal tissues, or in form of pro-vitamin A carotenoids such as β-carotene from plant tissues. Pro-vitamin A carotenoids are converted into active vitamin A (retinol) by β, β'-carotene 15-15'-monooxygenase (BCMO) [formerly known as β-carotene 15-15'-dioxygenase when discovery, later confirmed as a monooxygenase (Leuenberger et al., 2001)] in intestinal mucosa cells (Goodman and Kadis, 1965; Lindqvist and Andersson, 2002; Lindqvist and Andersson, 2004; Olson and Hayaishi, 1965). Although requirement of vitamin A can be fulfilled by a diverse diet, choice of food for people in developing countries is frequently limited to only cereal crops such as rice by productivity and/or cultural reasons. This makes vitamin A deficiency (VAD) a great health problem in those countries.

In combating VAD, the most promising strategy is believed to via engineering provitamin A fortified crops. Creation of golden rice signatures a significant

breakthrough in biotechnology for constructing a whole carotenoid biosynthetic pathway in the rice endosperm through genetic engineering (Ye et al., 2000). By introducing a daffodil phytoene synthase (*Psy*) and a bacterial carotene desaturase (*CrtI*) into the rice genome (Figure 1), carotenoid level in the endosperm was increased from nearly none to about 2 ug/g (Ye et al., 2000). PSY catalyzes the formation of phytoene by condensation of two molecules of geranylgeranyl diphosphate (GGPP). This is the first committed and also rate-limiting step in the carotenoid biosynthetic pathway. CRTI desaturates and isomerizes phytoene into the red colored lycopene. This CRTI cloned from bacteria is capable to substitute activities of four rice enzymes , which are PDS, ZDS, Z-ISO and CRTISO (Figure 1). Coexpression of PSY and CRTI in transgenic rice endosperms allowed accumulation of carotenoids beyond lycopene. Downstream products of lycopene such as α-carotene, β-carotene, lutein and zeaxanthin, were detected in the transgenic endosperms (Paine et al., 2005; Ye et al., 2000). This indicates endogenous enzymes such as β-carotene hydroxylase (βCHY) and ξ-carotene hydroxylase (ξCHY) (Figure 1) responsible for downstream reactions are present and enzymatically active in the rice endosperm.

Expression profiles of all the carotenogenic genes in developing rice endosperm have been analyzed by real time PCR. All the genes involved in the pathway are expressed in the endosperm, although *Psy* expression is extremely low to near to the detection limit (Schaub et al., 2005). Expressing *Psy* only in the rice endosperm resulted in accumulation of phytoene but not the downstream carotenoids (Burkhardt et al., 1997), and introduction of the bacterial *CrtI* alone did not result in any alternations in endosperm carotenoid content or composition (Schaub et al., 2005). Only when PSY and CRTI activities were combined

completes the pathway and without interferences to expression levels of other endogenous carotenogenic genes (Schaub et al., 2005). These results suggest that the low carotenoid content in the rice endosperm was attributed to low expression or low activity of PSY1 and one or more genes in the desaturation/isomerization system (system composed of four enzymes including PDS, ZDS, Z-ISO and CRTISO).

While much of the attentions have been focused on carotenoid biosynthesis, carotenoid catabolism in the endosperm is obscure. Several lines of evidence strongly suggested that members of carotenoids cleavage dioxygenase (CCD) family may play a role in carotenoid turnover in seeds. *In vitro* activity assay demonstrated CCD members cleaved specific double-bonds of different carotenoids to yield apocarotenoids. CCD1 showed a broad spectrum of substrate specificity on multiple double bonds (Ilg et al., 2009; Schwartz et al., 2001; Vogel et al., 2008). In *Arabidopsis*, loss of *AtCCD1* function in T-DNA insertion mutant resulted in increment of seed carotenoid content, while no significant alternations were detected in leaves (Auldridge et al., 2006).

Studies in maize provided further evidences for the role of CCD1 in carotenoid catabolism. In the study of maize White cap (*wc*) mutants, ZmCCD1 encoded by White cap locus (*Wc*) is found to be responsible for carotenoid breakdown in maize endosperm in a manner of dosage dependent (Tan et al., unpublished data) (Timmermans et al. 2004). High expression of *ZmCCD1* resulted in disappearance of yellow pigmentation in kernels, whereas loss of expression resulted in fully yellow kernels (Tan et al., unpublished data). Hi-II is a white endosperm line derived from A188 that is proven to contain the Wc allele (Tan et al., unpublished

data).

When cartenogenic genes *CrtB* and *CrtI* were expressed in Hi-II, expressions driven by γ-zein promoter failed to accumulate carotenoids. Only the plants that had their transgenes driven by a double γ-zein promoter were able to accumulate significant amount of carotenoids (Aluru et al., 2008). The active carotenoid catabolism in transgenic Hi-II provides a conceivable explanation for the compromised carotenoid accumulation. Furthermore, carotenoid accumulations were only be possible under strong expression of *CrtB/CrtI* (Aluru et al., 2008). This suggested carotenoid turnover by Wc can be saturated, although the amount was not determined.

In addition to CCD1, other CCD members are also potential candidates for carotenoid catabolism in the endosperm. These include CCD4, CCD7 and CCD8. Genome sequencing indicated CCD family is highly conserved among species although the biological functions of CCD1 and CCD4 are currently unknown. Four CCD members are found in rice, they are OsCCD1 (Os12g0640600), OsCCD4a (Os02g0704000), OsCCD4b (Os12g0435200), OsCCD7 (Os04g0550 600), OsCCD8a (Os01g0566500) and OsCCD8b (Os01g0746400). In this study, a combination of molecular, genetic and biochemical analyses were applied to address biological functions of these CCDs, especially in carotenoid catabolism in rice endosperm. Results revealed that OsCCD1 plays a predominant role in seed carotenoid turnover with high expression level, as well as strong *in vivo* enzymatic activity on a variety of carotenoids. To further the study, *OsCCD1* expression were knocked-down in both WT and Golden rice background, and thus its effects on seed carotenoid accumulations were examined.

Chapter 2. Literature review

2.1. Carotenoids in plants

Carotenoids are a diverse group of hydrophobic C40 isoprenoids usually red, yellow or orange in color. They are widely distributed in the nature. More than 750 members have been found naturally-occurring in plants, algae, cyanobacteria and some of the non-photosynthetic bacteria, fungi and some animals (Britton et al., 2004). According to chemical structures, carotenoids are divided into carotenes and xanthophylls. Carotenes are hydrocarbons in linear (such as lycopene) or cyclic forms at one or both ends (such as α-carotene and β-carotene). Xanthophylls are oxygenated hydrocarbons such as lutein, zeaxanthin, violaxanthin, antheraxanthin and neoxanthin.

In plants, carotenoids have multiple important functions that involve photosynthesis, photoprotection, substrates for plant hormones and aroma production. In photosynthesis, they are components of the chlorophyll-a-containing antenna complex and associated with reaction centers in photosynthesis system I and II (Baroli and Niyogi, 2000; Barros and Kuhlbrandt, 2009). Physically located in thylakoid membrane, carotenoids like violaxanthin, lutein and β-carotene act as accessory pigments to absorb light in blue and green regions of visible spectrum (420-480nm), and transfer the absorbed energy to chlorophylls in form of singlet electron excitation (Cogdell et al., 1994; Frank and Cogdell, 1996). This increases the efficiency of photosynthesis by broadening the light spectrum absorbed in the photosynthesis.

Although sunlight is the source of energy for plants, too much is harmful. Under intense sunlight, excessive excitation energy will be accumulated in light harvesting complex (LHC), leading to production of highly reactive oxygen species that will result in photoinhibition and oxidative damage to photosynthetic apparatus and adjacent cells (Demmig-Adams and Adams, 1992; Niyogi, 1999). To avoid this, carotenoids play a critical role in photoprotection. Actions of carotenoids include quenching of triplet state of chlorophylls and removal of the highly reactive singlet oxygen by non-photochemical quenching of chlorophyll fluorescence (NPQ) (Frank and Cogdell, 1996; Krinaky, 1979; Muller et al., 2001). NPQ involves the reversible conversion of violaxanthin to zeaxanthin via the "xanthophyll cycle". Under high light, excessive excitation energy is transferred from the singlet chlorophyll to violaxanthin. Then violaxanthin is de-epoxidated into zeaxanthin via the intermediate antheraxanthin under low luminal pH (Demmig-Adams and Adams, 1992) (refer to Figure 1). This whole process of xanthophyll cycle is reversible under low light. Functions of carotenoids in photosynthesis and photoprotection have been widely reviewed by different authors since its discovery (Baroli and Niyogi, 2000; Dreuw et al., 2005; Frank and Cogdell, 1996; Howitt and Pogson, 2006; Niyogi, 1999).

Not only are carotenoids important for direct participation in NPQ, carotenoid cleavage products, i.e. apocarotenoids, are biologically important as well. Besides aromas and flavors, carotenoids serve as the precursors of phytohormone abscisic acid (ABA) and strigolactone. ABA is derived from 9-*cis*-epoxycarotenoids (9-*cis*-violazanthin and 9-*cis*-neoxanthin). When they are cleaved by NCEDs at C11-C12 double bond, C15 xanthoxin would be formed and be subsequently converted to ABA (Schwartz et al., 1997a; Tan et al., 2003). On the other hand,

strigolactone is a newly identified plant hormone that functions as regulator for lateral bud formation (Gomez-Roldan et al., 2008; Umehara et al., 2008). It is derived from oxidative cleavage of carotenoids mediated by CCD7 and CCD8 (Lopez-Raez et al., 2008; Matusova et al., 2005; Schwartz et al., 2004). Until now, exact biosynthetic pathway of strigolactone is not clear yet.

Carotenoids also contribute to reproduction. Mature flowers and fruits store large amount of carotenoids in the chromoplast (Howitt and Pogson, 2006). The bright pigmentation of carotenoids in flowers visually attracts insects for pollination and animals for seed dispersal. Different plant species accumulate different types of carotenoids. For example, in potato root or tubers, major carotenoids presented are β-carotene, β-cryptoxanthin and lutein. In fruits and flowers, they are the lycopene in tomato, β-carotene in grape, mango and cashew apple. In seeds of plants such as canola, *Arabidopsis*, maize, wheat and sunflower, the major carotenoid presented is lutein. Depending on the weather during cultivation, carotenoid contents will varies among different individuals even they belong to the same species. Detailed information were summarized in a recent review (refer to Howitt and Pogson, 2006).

2.2 Carotenoid biosynthesis in plants

In plants, carotenoid biosynthesis takes place in nearly all kinds of plastids. These include the colored chromoplasts, starch-storing amyloplasts, lipid-storing elaioplasts, chloroplast precursor etioplasts and photosynthetic chloroplasts (Howitt and Pogson, 2006). Carotenoid biosynthetic pathway involves three major steps: desaturation, isomerization and cyclization (refer to Figure 1). The biosynthetic genes have been well characterized (Cunningham and Gantt, 1998;

Hirschberg, 2001). The whole pathway starts with the condensation of two C20 geranylgeranyl diphosphate (GGPP) molecules to form 15-*cis*-phytoene, which is a colorless C40 carotenoid. This reaction is found to be the rate-limiting step of the pathway and is catalyzed by phytoene synthase (PSY). There are three isoforms of PSY in rice, in which OsPSY1 and OsPSY2 are involved in carotenogenesis in photosynthetic tissues, whereas OsPSY3 is associated with abiotic stress-induced ABA formation (Li et al., 2008; Welsch et al., 2008). Phytoene is further desaturated into the red colored lycopene by two structurally and functionally alike enzymes, phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS). As phytoene itself is in poly-*cis* configuration, whereas lycopene is in all-*trans* configuration, conversion of *cis* to *trans* configuration is essential. The isomerization is catalyzed by 15-*cis*-ξ-CRTISO (Z-ISO) and carotene-isomerase (CRTISO). Each of them isomerizes lycopene at different positions (Isaacson et al., 2004; Isaacson et al., 2002; Li et al., 2007; Li et al., 2008; Park et al., 2002). Desaturation of phytoene and isomerization of lycopene are catalyzed by four enzymes in cyanobacteria and higher plants. In contrast, in fungi and bacteria such as *Erwinia stewartii*, only a single enzyme carotene desaturase (CRTI) is able to catalyze all of the four reactions (Bartley et al., 1999; Sandmann, 1994).

Cyclization of all-*trans* lycopene results in two branches: one branch forms β-carotene and its derivative zeathxanthin; another branch forms α-carotene (β, ε-carotene) and its derivative lutein. β-carotene is formed by the introduction of one β-ring at both ends of a linear lycopene molecule, which involves a two-step reaction catalyzed by lycopene β-cyclase (LCYβ). α-carotene is formed by the introduction of one ε-ring and one β-ring at each end of the lycopene respectively,

and this involves reactions catalyzed by lycopene ε-cyclase (LCYε) and lycopene β-cyclase (LCYβ) sequentially (Hirschberg, 2001; Lu and Li, 2008). Relative activities of LCYβ and LCYε are found to have roles in regulating the ratio of α-carotene/ β-carotene formation (Harjes et al., 2008). Knockdown/knockout expression of LCYε in potato tubers (Diretto et al., 2006) , *Arabidopsis* (Pogson et al., 1996) and *Brassica* seeds (Yu et al., 2008) was found to cause increments in β-carotene and downstream carotenoids accumulations, but without obvious alternation in α-carotene formation.

By a series of hydroxylation, epoxidation and isomerization, α-carotene and β-carotene can be further modified into different forms of carotenoids. Introduction of hydroxyl (-OH) groups into α-carotene and β-carotene leads to the formation of lutein (Kawakatsu et al., 2008; Lu and Li, 2008) and zeathxanthin (Lu and Li, 2008), respectively. Lutein is the most frequently presented carotenoid in green tissues of plants (Hirschberg, 2001), whereas zeathxanthin has important role in protecting plants from high light stress through "xanthophyll cycle" (Demmig-Adams and Adams, 1996; Dreuw et al., 2005; Eskling et al., 1997). Further down the carotenoid biosynthetic pathway, violaxanthin is converted into neoxanthin by neoxanthin synthase (NSY) (North et al., 2007). Formation of neoxanthin is considered as the final step of classical carotenoid biosynthetic pathway. In the downstream reactions, nine-*cis*-epoxycarotenoid dioxygenase (NCED) is able to cleave 9-*cis*-neoxanthin and/or 9-*cis*-violaxanthin into xanthoxin, which is the immediate precursor of abscisic acid (Burbidge et al., 1999; Schwartz et al., 1997b; Tan et al., 1997).

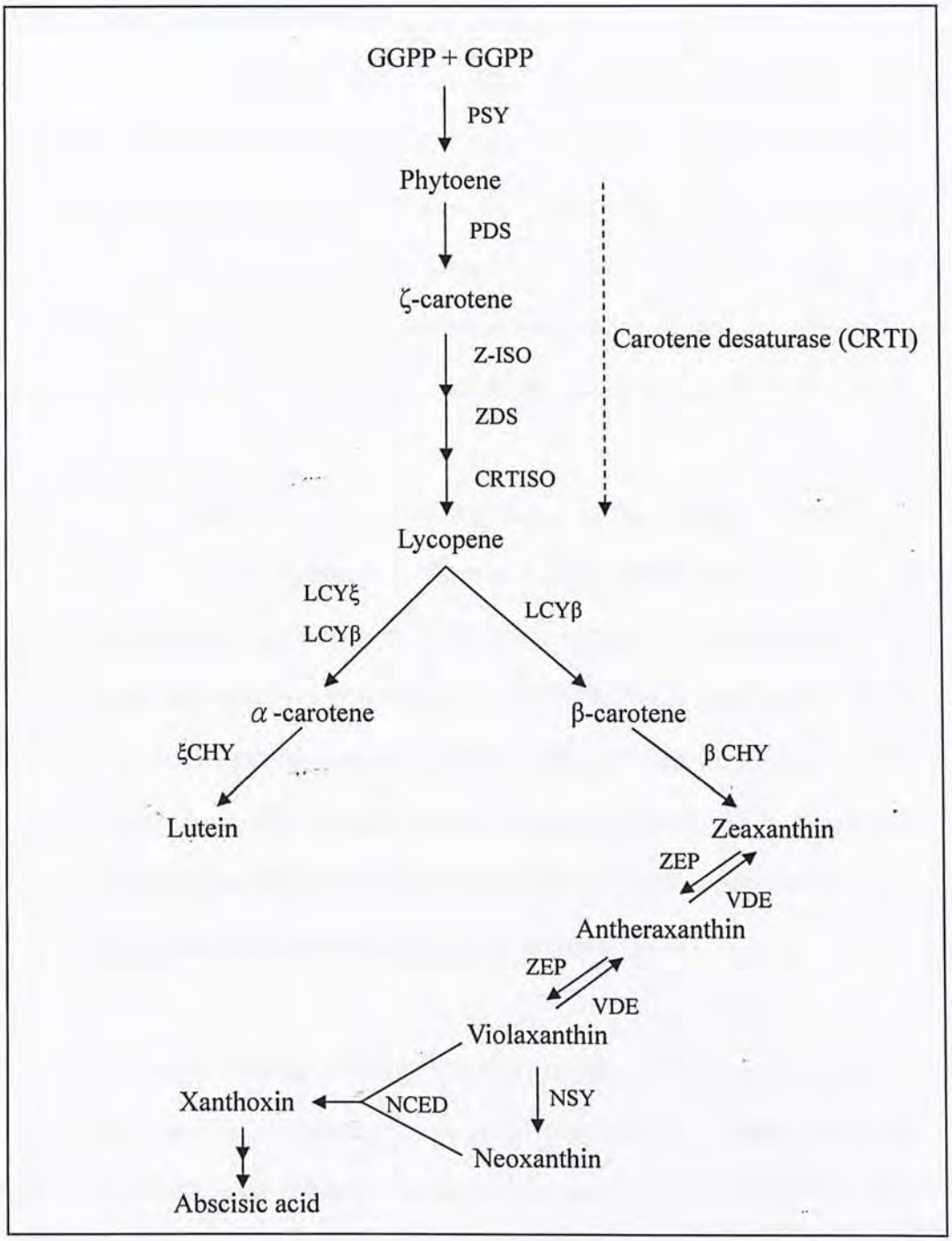

Figure 1. Simplified carotenoid biosynthetic pathway in plants. GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-cis-ξ-carotene isomerase; ZDS, ξ-carotene desaturase; CRTISO, carotene isomerase; LCYβ, lycopene β-cyclase; LCYξ, lycopeneξ-cyclase; βCHY, β-carotene hydroxylase; ξCHY, ξ-carotene hydroxylase. ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase ; NCED, nine-*cis*-epoxycarotenoid dioxygenase.

2.3. Carotenoids in animals

Carotenoids have health benefits to animals and human, especially as antioxidants, precursors of vitamin A and regulator of immune system (reviewed by Fraser and Bramley, 2004; Krinsky and Johnson, 2005; Moise et al., 2005; Rao and Rao, 2007). Intake of carotenoids such as lycopene, β-carotene, lutein and zeaxanthin is also found to lower the risk of certain cancers, heart disease and age-related macular degeneration (Cooper et al., 1999; Landrum and Bone, 2004).

The most well known function of carotenoids is as the precursor of vitamin A (also known as "Pro-vitamin A"). Vitamin A (also refer as to retinol) and its derivatives retinoids are essential nutrients for growth, eye development and maintenance of immune system (Fierce et al., 2008; Yeum and Russell, 2002). Animals, including human, cannot synthesize vitamin A *in vivo*, and hence must be obtained from the diet. Dietary sources of vitamin A are in two forms, in form of retinyl ester (long chain fatty acid esters of retinol) from animal sources and in form of pro-vitamin A carotenoids from plant sources.

Animal source of vitamin A is often in form of long chain fatty acid esters of retinol, and must be hydrolysed in the small intestine lumen before absorption (Harrison, 2005). After ingestion, dietary retinyl ester was released from the food matrix, solubilized and incorporated into water-miscible micelles in the presence of bile salts (Institute Of Medicine, 2001; Parker, 1996). Then, together with triglycerides, retinyl ester is hydrolysed by pancreatic triglyceride lipase (PTL) in the intestinal lumen (van Bennekum et al., 2000) and phospholipase B (PLB) that associated to the brush border of enterocyte (Rigtrup et al., 1994). After that, they are absorbed by mucosal cells as free alcohol retinol (Dew and Ong, 1994). As

retinol is hydrophobic in nature, high fat diet was found to facilitate the uptake of retinol in mucosal cells (Ribaya-Mercado, 2002; Tso et al., 2001). Within enterocytes, newly absorbed retinol is re-esterified with fatty acid by acyl-CoA acyltransferase (ARAT) (Ross, 1982) and lecithin retinol-acyl transferase (LRAT) (O'Byrne et al., 2005). After that, they are packaged into chylomicrons with neutral lipid esters such as triacylglycerol and cholestyl esters (Harrison, 2005). Chylomicrons are then secreted into the lymphatic system (Harrison, 2005; Harrison and Hussain, 2001) for storage in liver and metabolism in other parts of the body when needed.

Plant derived vitamin A sources are carotenoids in fruits and vegetables. They are restricted to only about fifty members of carotenoids that contain at least one unsaturated β-ionone ring. These include α-carotene, β-carotene, zeaxanthin and β-cryptoxanthin, in which, β-carotene has the highest pro-vitamin A conversion efficiency (Fraser and Bramley, 2004). Upon uptake, in the intestinal mucosal cells, dietary β-carotene can be cleaved symmetrically at central carbon 15, 15' position by β, β'-carotene 15, 15' monooxygenase (BCMO1; EC 1.14.99.36), a cytosolic non-heme iron-containing enzymes, and results in two molecules of retinal (retinaldehyde). Retinal will then be reversibly reduced into retinol (vitamin A) by retinal reductase, or irreversibly oxidized into retinoic acid by retinal dehydrogenase (Parker, 1996; Roberts and Deluca, 1967; van Vliet et al., 1995). Retinol formed in this way will be esterified in the enterocytes, and transported to the liver in chylomicrons for storage.

The key step of vitamin A biosynthesis requires cleavage at central position of pro-vitamin A carotenoids (Goodman et al., 1966; Olson and Hayaishi, 1965). Yet,

this took scientists 50 years to find out the identity of enzyme responsible for this reaction. Based on the sequence homology to VP14 [the nine-*cis*-epoxycarotenoid dioxygenase (NCED) responsible to cleave 9-*cis*-neoxanthin and/or 9-*cis*-violaxanthin to form ABA in maize], cDNA encoding for animal vitamin A biosynthesis-related enzyme, β, β'-carotene 15, 15' monooxygenase (BCMO1), was first cloned and sequenced from *Drosophila melanogaster* (von Lintig and Vogt, 2000) and chicken (Wyss et al., 2000; Wyss et al., 2001). This breakthrough allowed study of vitamin A biosynthesis from carotenoids at molecular level. Although BCMO1 was thought to be a dioxygenase, it was later proven to be a monooxygenase instead (Leuenberger et al., 2001). BCMO1 works on pro-vitamin A carotenoids in three steps: epoxidation of the C15- C15' central double bond, followed by unselective ring opening by hydration, then oxidative cleavage at the diol formed previously (Leuenberger et al., 2001). Throughout these steps, only one oxygen atom from water and/or air was incorporated into the cleavage product. After the identification of BCMO1, its homologues have been identified in different animals such as mouse, rat (GenBank accession number: NM_053648), chicken (Wyss et al., 2000), zebrafish (GenBank accession number: AJ290390) and human, in which, human BCMO1 was found to be expressed especially high in liver and intestine (Lindqvist and Andersson, 2002).

2.4. Vitamin A deficiency (VAD)

Vitamin A is an essential nutrient for maintaining vision and health. Deficiency of vitamin A can cause multiple health consequences, affecting especially pre-school children and pregnant women. VAD weakens immune system, exacerbates diseases like measles, anemia, diarrhea and general infectious diseases; hinders the development of eye, causes eye diseases ranged from night blindness,

keratomalacia (dry cornea), xerophthalmia [failure to produce tears; the number one cause of preventable blindness in children (Sommer and West, 1996)] or even complete blindness.

In at least 26 developing countries, VAD has been a great problem, especially for countries in South Asia and Africa, where carotenoid-low cereals like rice grains are consumed as staple food. According to recent WHO (World Health Organization) statistics, 250 to 300 thousands VAD children become blind each year. And about 160 thousands children and women are killed by VAD-causing or VAD-related diseases. (Details of statistics data of individual country please refer to WHO Global Database of Vitamin A deficiency, http://www.who.int/vmnis/vitamina/en/).

To access VAD status in a population, two sets of indicators have been used by WHO. Diagnosed stages of xerophthalmia defined by WHO has been regarded as clinical indicator, they are night blindness (XN), Bitot's spots (X1B), and active corneal disease (X2 and X3) (Sommer, 1995). For the second indicator, retinol concentration in blood serum/ plasma has been regarded as a biochemical parameter for vitamin A content in the body. A cut-off line of 0.70 μmol/L is used by WHO to represent the occurrence of VAD (WHO, 1996). If the value is under 0.35μmol/L, the person is said to be in the severe state of VAD. For the case of pregnant and lactating women, due to their higher vitamin A requirement, cut-off line of VAD increases to 1.05μmol/L (West, 2002).

2.5. Recommended requirement of vitamin A

In order to maintain health and prevent VAD, it is necessary to intake sufficient amount of vitamin A from the diet. For easy planning and managing the dietary content, three different nutrition labeling systems have been established, so as to express dietary vitamin A activity in a common unit. They are "International Unit "(IU), "Retinol Equivalent" (RE) and "Retinol Activity Equivalent" (RAE). "International Unit "(IU) is the oldest parameter among all. 1 IU is equivalent to 0.3μg of dietary retinol, 0.6μg of β-carotene in mixed diet or 1.2 μg of other pro-vitamin A carotenoids (WHO and FAO, 2004). Since IU do not consider the conversion efficiency of pro-vitamin A into retinol, it is difficult to calculate the actual amount of vitamin A in the diet. Seeing this, a "Retinol Equivalent" (RE) system has been established by a Joint WHO/FAO Expert Group in 1967. Relationship between retinol and dietary pro-vitamin A carotenoids have been established, 1RE is equivalent to 10 IU of β-carotene, 1μg of dietary retinol, 2μg of β-carotene supplement dissolved in oil, 6μg of β-carotene in a mixed diet or 12μg of other pro-vitamin A carotenoids (WHO, 1967). As diet composition can affect bioconvertibility of dietary pro-vitamin A carotenoids, variations in the conversion efficiency can be observed in different countries with different dietary habits. In 2001, a new unit, "Retinol activity equivalent" (RAE), was established by the Food and Nutrition Board of the Institute of Medicine (USA). Compared with countries such as Holland where diet largely composed of green leafy vegetables, in USA where fruits occupy a large portion of diet, lower conversion rate of pro-vitamin A into retinol is observed (WHO and FAO, 2004). Unlike RE, 1 RAE is equivalent to 1μg of dietary retinol, 2μg of β-carotene supplement dissolved in oil, 12μg of β-carotene in a mixed diet or 24μg of other pro-vitamin A carotenoids (Institute Of Medicine, 2001).

Requirement of vitamin A varies among different sexes and different age groups. In general, daily requirement of vitamin A increases with ages, and men requires more vitamin A than women, except pregnant and lactating women who require the highest amount of retinol per day among all groups (table 1).

Table 1. Estimated mean requirement and safe level of intake for vitamin A, by groups. (Adopted from "Vitamin and mineral requirements in human nutrition: report of a joint FAO/WHO expert consultation" by World Health Organization. 2004. Pp. 35)

Group		Mean requirement (μg RE per day)	Recommended safe intake (μg RE per day)
Infants and children	0-6 months	180	375
	7-12 months	190	400
	1-3 years	200	400
	4-6 years	200	450
	7-9 years	250	500
Adolescents	10-18 years	330-400	600
Adults (Females)	19-65 years	270	500
	65+ years	300	600
Adults (Males)	19-65 years	300	600
	65+ years	300	600
Pregnant women		370	800
Lactating women		450	850

Abbreviations: Mean requirement, the minimum daily requirement of vitamin A so as to prevent VAD-caused xerophthalmia; RE, retinol equivalent.

2.6. Bioavailability and bioconversion of dietary carotenoids

Dietary carotenoids from plants are one of the major vitamin A sources for human. Uptake and utilization of dietary carotenoids can be affected by at least nine factors. They were summarized by Castenmiller and West (1998) into a mnemonic, "SLAMENGHI", which are "(1)Species of carotenoids (2) Molecular linkage (3) Amount of carotenoids consumed in the diet (4) Matrix in which the carotenoids is incorporated (5) Effectors of absorption and bioconversion (such as dietary fat, dietary fiber and alcohol) (6) Nutrient status of the host (7) Genetic factors (8) Host related factors (such as sex, age and health status) (9) Mathematrical Interactions (interactions between two or more factors)" (Castenmiller and West, 1998). Among all these factors, factors related to bioavailability and bioconversion efficiency into retinol and host health status are considered to be the most influential ones.

By definition, bioavailability refers to the portion of diet absorbed and utilized in physiological functions or storage (Maiani et al., 2009). Carotenoid species in the diet, composition of food matrix and host health can be major factors to taken into account.

In general, carotenoids are synthesized in plants in *cis*- and *trans*-isoforms. Although they both exist in the plants, all-*trans*-β-carotene is found to be absorbed and converted into retinol more readily than β-carotene in *cis*-configuration (Castenmiller and West, 1998; Gaziano et al., 1995; Jensen et al., 1987). Ratio of *trans*- and *cis*-carotenoids varies between different plant species. However, in the process of cooking, *tran*- to *cis*- isomerization and oxidation of β-carotene and lycopene were observed in vegetables such as tomatoes and carrots

(Bernhardt and Schlich, 2006; Mayer-Miebach et al., 2005; O'Sullivan et al., 2010; Rodriguez-Amaya, 1999). Presence of suitable amount of oil in the diet could enhance β-carotene absorption (Tso et al., 2001) whereas too much dietary fiber, such as pectin, may reduce the absorption efficiency of carotenoids (Riedl et al., 1999; Rock and Swendseid, 1992). In addition, health status of individuals could affect efficiency of carotenoids absorption. Although relationship between carotenoids absorption and malaria was not clear, plasma carotenoids and retinol concentration in malaria patient was significantly lower than that in healthy person (Das et al., 1996; SanJoaquin and Molyneux, 2009).

For bioconvertibility, it refers to the ratio of dietary carotenoids able to convert to retinol in the body. Upon absorption, each molecule of β-carotene would be cleaved symmetrically at central carbon by BCMO1 (Leuenberger et al., 2001; von Lintig and Vogt, 2000). Conversion ratio of dietary β-carotene into retinol was estimated to be 2:1. However, in clinical studies, this ratio can only be achieved when purified β-carotene was supplied in oil (Sauberlich et al., 1974). For mixed diet containing large amount of green leafy vegetables, 7 μg of dietary β-carotene is equivalent to 1μg of purified β-carotene supplied in oil, while for other pro-vitamin A carotenoids (such as lutein), 14μg of them would be equivalent to 1μg of β-carotene supplied in oil (van het Hof et al., 1991). So, the conversion factor of dietary β-carotene into retinol would be 14:1 [Retinol activity equivalents (RAE)= 14:1], for other pro-vitamin A carotenoids, that would be 28:1 (WHO and FAO, 2004). As bioavailability of carotenoids can be affected by the composition of diet, for regions like USA where fruits occupied large portion of the diet, it has been reported that the conversion factor of dietary β-carotene to retinol is 6:1, instead of 7:1, whereas for other pro-vitamin A carotenoids, it would

be 12:1 (Institute Of Medicine, 2001). Differences in conversion ratios may be due to the accessibility of carotenoids during digestion. In green leafy vegetables, carotenoids are mainly located in the chloroplasts associated with pigments in form of pigment-protein complex. It may be difficult to release carotenoids from the complex during digestion, causing the low conversion factor for diet composed largely with vegetables. For the case in fruits, carotenoids such as β-carotene are located in chromoplast or lipid droplet, which make carotenoids easier to be released from the food matrix during digestion.

2.7. Efforts to improve carotenoid contents in food crops

To overcome the problem of VAD, one of the promising solutions is to increase pro-vitamin A carotenoid accumulation in staple crops such as rice, a staple food providing calories for more than half of the world's population. The strategy has been focused on increasing the biosynthetic activity through genetic engineering. After the successfulness in creating golden canola seeds in 2002 (Shewmaker et al., 2002), high carotenoid accumulation has been achieved in different transgenic food crops . The most famous example is the "Golden rice" (Ye et al., 2000). By introducing daffodil phytoene synthase (PSY) and bacterial phytoene desaturase (CRTI), Golden rice was created that elevated the carotenoid content from almost none to 1.6 μg/g in rice seeds (Ye et al., 2000). Further modification using maize PSY led to a further increment of carotenoids up to about 37 μg/g in rice seeds (Paine et al., 2005). By overexpressing other enzymes involved in the carotenoid biosynthetic pathway, "Golden" potato and high-carotenoid- maize have been created (Diretto et al., 2007; Diretto et al., 2006; Zhu et al., 2008).

2.8. Carotenoid catabolism

While many attentions have been focused on increasing biosynthesis of carotenoids, the potential catabolism received little attention. Carotenoids, like other metabolites, are subjected to catabolism. Oxidative cleavage products of carotenoids known as apocarotenoids are produced by oxidative cleavage at specific bonds on polyene chain. Certain apocarotenoids are biological active regulators which include phytohormone ABA in plants and vitamin A in animals. A newly discovered plant hormone, strigolactone, which regulates branching formation was believed to be derived from cleavage of carotenoids as well (Gomez-Roldan et al., 2008; Matusova et al., 2005; Umehara et al., 2008). In addition, aromatic apocarotenoids exert functions in reproduction as pollinator attractants (Fraser and Bramley, 2004; Hirschberg, 2001; Moise et al., 2005). Some of the apocarotenoids even have economical values. Bixin (annatto) from *Bixa orelloma* (Bouvier et al., 2003a) and crocin from *Crocus satius* (saffron) are two of the representatives (Bouvier et al., 2003b). Bixin is the pigment commonly used in cosmetics and as food color additives since pre-Colombian times (Bouvier et al., 2003a). For crocin, it is the major component responsible for the pigment of saffron, the most expensive spice in the world (Bouvier et al., 2003b).

2.9. Carotenoid cleavage dioxygenase (CCD)

Double bond-specific oxidative cleavages of carotenoids are carried out by carotenoid cleavage dioxygenase (CCD) enzymes. Founding member of CCD family is VP14 (VIVIPAROUS14), which is identified in a maize abscisic acid (ABA) deficient mutant (Tan et al., 1997). VP14 is a key regulatory enzyme in the ABA biosynthetic pathway. It cleaves 9-*cis* neoxanthxin and/or 9-*cis* violaxanthin asymmetrically at C11-C12 double bond, leading to the generation of C15 xanthoxin. Xanthoxin is the immediate precursor of ABA (Figure 1) (Tan et al., 2003). Due to its substrate requirement of 9-*cis* epoxycarotenoids, VP14 is classified as a nine-*cis*-epoxy carotenoid cleavage dioxygenase (NCED). Based on sequence similarity, homologous genes of *Vp14* have been identified in a varieties of organisms, including fungi, plants such as *Arabidopsis*, cyanobacteria and bacteria such as *pseudomonas*, insects such as drosophila and animals such as human and mouse, etc. (Ho et al., 2007; Marasco et al., 2006; Paik et al., 2001; Prado-Cabrero et al., 2007a; Prado-Cabrero et al., 2007b; Redmond et al., 2001; Schwartz et al., 2001; Tan et al., 2003; von Lintig and Vogt, 2000). Examples of VP14 homologues were shown in a phylogenetic tree in figure 2. Sequences were aligned using ClustalW2 program on the internet (http://align.genome.jp).

All the CCD family members share two common characteristics. First, they all require ferrous ion to exert their functions. Removal of iron by iron-chelating agents will causes total loss of cleavage activities. The activities are restored by supplement of ferrous (Fe^{2+}) ion, but not Fe^{3+} ion (Lakshman et al., 1972; Lindqvist and Andersson, 2002; Moiseyev et al., 2006; Schwartz et al., 1997a). Secondly, although homology of amino acid sequences between different homologues may be low, all CCD members contain four highly conserved

histidine residues. These residues are proven to coordinate non-heme iron and oxygen binding (Kloer et al., 2005; Schwartz et al., 1997a; Tan et al., 1997). To keep the binding centre in correct geometry, these histidine residues are fixed by several glutamate and aspartate residues (Kloer and Schulz, 2006). These histidine residues are essential for enzymatic activity. Substitution of any one of these residues in mouse β-Carotene 15,15′-monooxygenase-1 (BCMO1) (Poliakov et al., 2005) and *Noctoc* sp. PCC7120 carotenoid cleavage dioxygenases (NSC1) (Marasco et al., 2006) resulted in total loss of activity.

A further classification of CCDs is based on the functional relationship with ABA biosynthesis. If the cleavage leads to the biosynthesis of ABA (such as the activity of VP14), the enzyme is further named as nine-*cis*-epoxycarotenoid dioxygenase (NCED) so as to indicate its specific substrate requirement and cleavage site. If the cleavage is not related to ABA biosynthesis, they are simply classified as carotenoid cleavage dioxygenase (CCD). All the plant CCD members are clustered into nine subfamilies according to sequence homologies to their representatives in *Arabidopsis thaliana.* There are nine CCD members in *Arabidopsis* of which five are NCEDs (AtNCED2, 3, 5, 6 and 9) (Iuchi et al., 2001; Tan et al., 2003). The remaining four members are CCDs (AtCCD1, 4, 7 and 8). They can cleave various *trans*-carotenoids at specific double bonds to produce apocarotenoids.

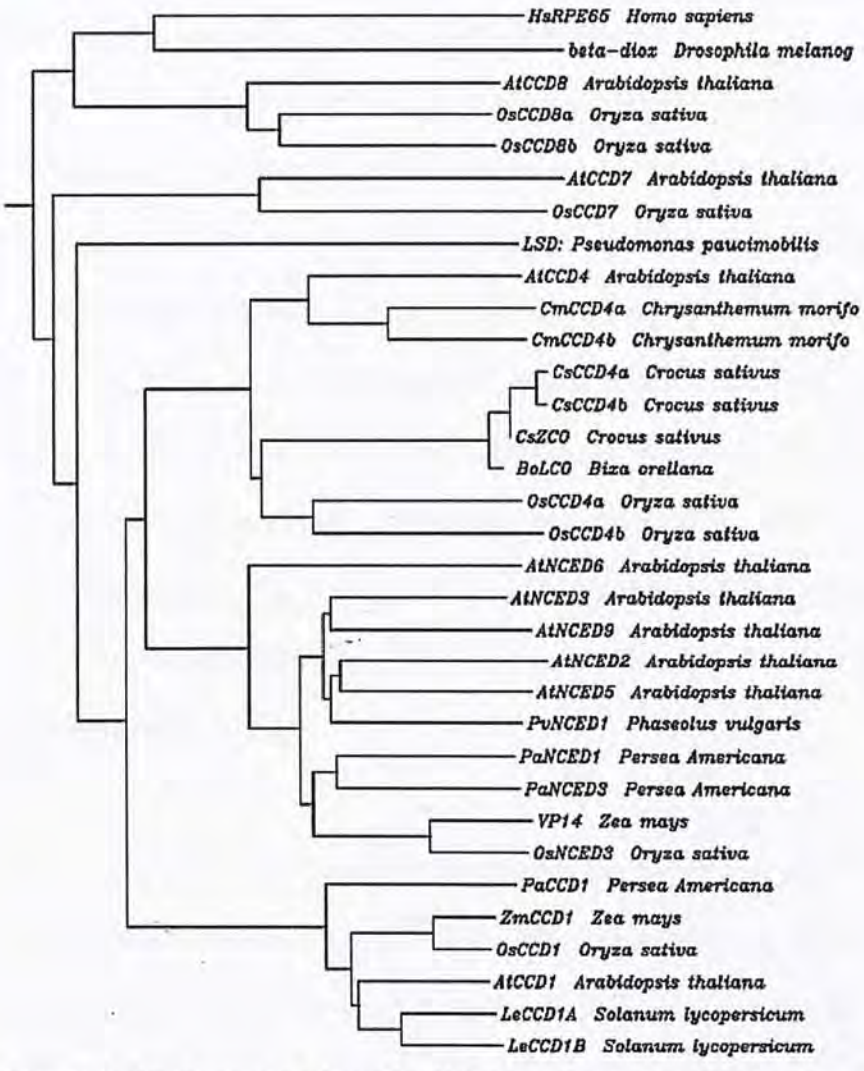

Figure 2. Phylogenetic tree of VP14 related genes in different organisms.

VP14 (NM_001112432) and ZmCCD1(DQ100346) of *Zea may*; AtCCD1(NM_116217), AtNCED2(NM_117945), AtNCED3(NM_112304), AtCCD4(NM_118036), AtNCED5(NM_102749), AtNCED6(NM_113327), AtCCD7(NM_130064), AtCCD8(NM_119434)and AtNCED9(NM_106486) of *Arabidopsis thaliana*; beta-diox(AJ276682) of *Drosophila melanogaster* ; BoLCO (AJ489277), lycopene cleavage oxygenase of *Bixa orellana*; CmCCD4a (AB247158) , CmCCD4b (AB247160) of *Chrysanthemum morifolium*; CsCCD4a (EU523662), CsCCD4b(EU523663), CsZCO (zeaxanthin cleavage oxygenase; AJ489276) of *Crocus sativus* ; HsRPE65(AF039855), retinal pigment epithelium-specific protein of *Homo sapiens*; LeCCD1A (AY576001)and LeCCD1B(AY576002) of *Solanum lycopersicum*; OsCCD1(NM_001073927), OsCCD4a (NM_001054393), OsCCD4b (NM_001073218), OsCCD7 (NM_001060026), OsCCD8a(NM_001049898), OsCCD8b (NM_001050764), OsNCED1(AY838897), OsNCED2(AY838898) and OsNCED3(AY838899) of *Oryza sativa*; PaNCED1(AF224672), PaCCD1(AF224670) and PaNCED3 (AF224671) of *Persea Americana*; LSD(S65040), lignostilbene-α, β-dioxygenase of *Pseudomonas paucimobilis*; PvNCED1 (AF190462) of *Phaseolus vulgaris.*

2.10. Carotenoid-derived phytohormones

Strigolactone is a carotenoid-derived phytohormone besides ABA. It is a class of compounds first characterized as chemical signals secreted from plant roots into rhizosphere. These compounds can stimulate the seed germination of root parasitic weeds such as *Striga* and *Orobanche* (Bouwmeester et al., 2003; Cook et al., 1972; Cook et al., 1966), and induce hyphae branching of symbiotic arbuscular mycorrhiza (AM) fungi (Akiyama et al., 2005). Although they were first regarded as sesquiterpene lactones (Akiyama et al., 2005), strigolactones were confirmed to be a group of terpenoid lactones derived from carotenoid biosynthetic pathway in a way similar to that of ABA (Lopez-Raez et al., 2008; Matusova et al., 2005).

Recently, studies of mutants with extensive branching phenotype allowed identification of a novel graft-transmissible signal involving in shoot branching inhibition (Foo et al., 2001). The signal was then confirmed to be strigolactone and its derivatives, and thus defined as a novel class of phytohormone (Gomez-Roldan et al., 2008; Umehara et al., 2008). These mutants include *rms (ramosus)* in pea (*Pisum sativum*) (Beveridge, 2000; Beveridge et al., 1994; Beveridge et al., 1996; Beveridge et al., 2000); *max (more axillary branching)* in *Arabidopsis* (Sorefan et al., 2003; Stirnberg et al., 2002; Turnbull et al., 2002); *d (dwarf)* and *htd/d* (*high-tillering dwarf/dwarf*) in rice *(Oryza sativa*) (Arite et al., 2007; Arite et al., 2009; Ishikawa et al., 2005; Zou et al., 2006); *dad (decreased apical dominance)* in petunia (*Petunia hybrid*) (Simons et al., 2007; Snowden et al., 2005) and *Solanum lycopersicon Orobanche-Resistant Trait 1* (*Sl-ORT1*) in tomato (Koltai et al., 2010).

Molecular characterization of these mutants identified *RMS5*, *MAX3*, *HTD1/D17*and *SlCCD7* encode for orthologues of CCD7 (Booker et al., 2004; Johnson et al., 2006; Vogel et al., 2010; Zou et al., 2006), whereas *RMS1*, *MAX4*, *D10*, *DAD1* encode orthologues of CCD8 (Arite et al., 2007; Snowden et al., 2005; Sorefan et al., 2003). CCD7 and 8 are found to be localized in plastids and act in the first few steps of strigolactone biosynthesis sequentially. With its actual substrate not yet identified, CCD7 is proposed to cleave C40 carotenoids such as β-carotene at C9-C10 double bond asymmetrically, leading to production of C27 apocarotenoids such as 10'-apo-β-carotenal (Schwartz et al., 2004). These intermediates are further cleaved by CCD8 at C13-C14 double bond into C18 and C9 apocarotenoids. C18 apocarotenoids such as 13-apo-β-carotenone are suggested to be the precursors of downstream reactions (Schwartz et al., 2004) (Figure 3).Recently, a possible third member in strigolactone biosynthetic pathway, D27, is identified (Lin et al., 2009) (Figure 3). It is a novel putative chloroplast-localized iron-containing protein with no annotated function, yet is essential to strigolactone biosynthesis (Lin et al., 2009).

Characterization of *Arabidopsis max1* mutant identified an oxidative modification step acting downstream of MAX3 (CCD7) and MAX4 (CCD8) (Booker et al., 2005), possibly as well as D27. This step is catalyzed by a cytochrome P450 family monooxygenase encoded by *MAX1*, and may be related to the activation of the mobile strigolactone although both substrate and product are not yet been identified (Beveridge and Kyozuka, 2010; Booker et al., 2005) (Figure 3).

Some of the extensively branching mutants could not be rescued by exogenous strigolactone, indicating that the genetic lesions are in the signal transduction or

perception instead of biosynthesis. These mutants include *Arabidopsis max2*, pea *rms4* and rice *d3* mutants. Based on sequence homology, *MAX2, RMS4* and *D3* are orthologues, and encoded for members of F-box leucine-rich repeat protein located at nodes/buds (Ishikawa et al., 2005; Johnson et al., 2006; Stirnberg et al., 2007; Stirnberg et al., 2002). F-box protein is commonly participate in transduction pathway of hormone, in which it acts as substrate-recognizing motif of SCF-type (Skp1-Cul1/Cdc53-F-box-type) ubiquitin E3 ligase so as to degrade specific regulator protein through the Ubiquitin/26S proteasome proteolytic pathway (Vierstra, 2003). Example can be observed in auxin signaling pathway. When auxin present, AUX/IAA protein that bind to auxin-response element such as ARF family of transcription factors will be recognized by SCF^{TIR1} (SCF complex containing TIR1 F-box protein as substrate-recognizing motif), and be degraded through Ubiquitin/26S proteasome proteolytic pathway (Zenser et al., 2001). Without the blocking of AUX/IAA proteins, auxin-responsive gene can be transcribed and perform their cellular functions (Hellmann and Estelle, 2002). With reference to auxin signaling pathway, strigolactone signal transduction is probably regulated by protein degradation in a way similar to that of auxin.

Recently, characterizations of three strigolactone-insensitive mutants provided additional information to strigolactone signaling pathway. These are *d14*, *d88* and *htd2* mutants of rice. *D14* encodes a protein which belongs to α/β-fold hydrolase superfamily whereas *D88* and *HTD2* are predicted to encode esterase and esterase/lipase/thioesterase family respectively (Arite et al., 2009; Gao et al., 2009; Liu et al., 2009). It is proposed that all these three genes are involved in strigolactone signaling pathway, however, the mechanisms are still unclear.

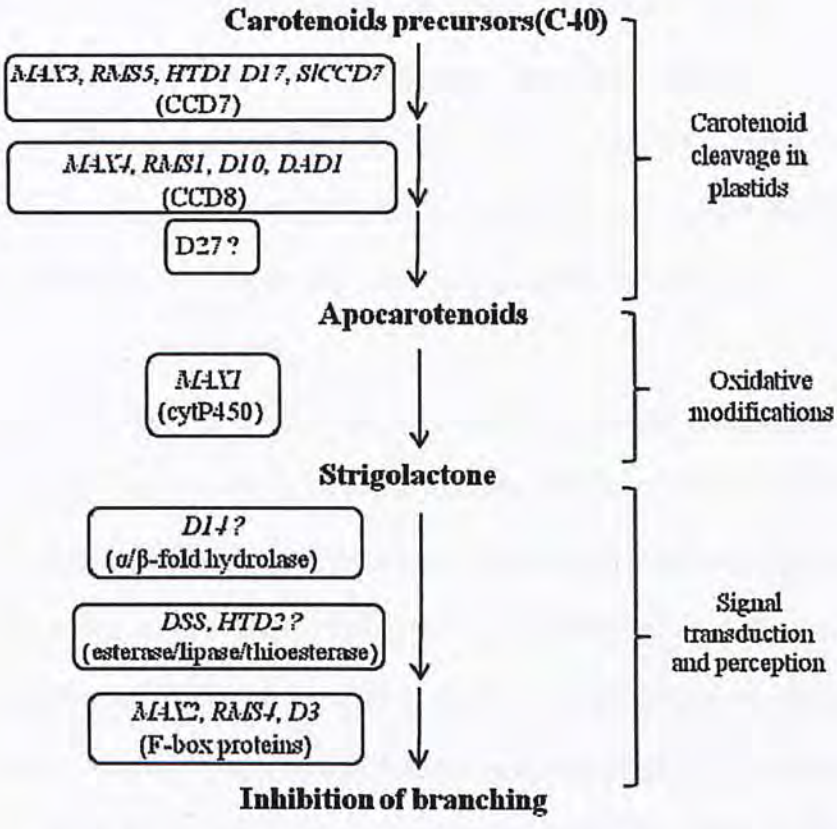

Figure 3. Proposed biosynthetic and signaling pathway of strigolactone

2.11. CCD and carotenoid-derived colors, aromas and flavors

Colors, aromas and flavors of flowers and fruits are often affected by carotenoid content. They involved site-specific oxidative cleavage of carotenoids by two subclasses of carotenoid cleavage dioxygenase, CCD1 and CCD4.

Carotenoid Cleavage Dioxygenase 1(CCD1)

CCD1 is the best studied CCD in plants. In *Arabidopsis*, it is the only CCD member that does not contain any plastid-targeting signal (Tan et al., 2003), and was found to be localized in cytoplasm, whereas the other CCD members are localized in plastids (Auldridge et al., 2006; Bouvier et al., 2003b). CCD1 was found to have a broad spectrum of substrate specificity and cleavage sites.

Substrates of CCD1 included both linear (ζ-carotene and lycopene) and cyclic carotenoids (β-carotene, zeaxanthin and δ-carotene) (Schwartz et al., 2001; Vogel et al., 2008). However, CCD1 did not cleave phytoene, the first carotenoid formed in carotenoid biosynthetic pathway (Vogel et al., 2008), nor geranylgeranyl diphosphate (GGPP), the precursor for carotenoid biosynthesis.

The cleavage activity of CCD1 was first discovered in *Arabidopsis*. Recombinant AtCCD1 was found to cleave a number of carotenoids at C9-C10 (C9'-C10') bonds symmetrically *in vitro*, producing two C13 ketones and one C14 aldehyde (Schwartz et al., 2001). This cleavage activity was confirmed by analyzing orthologous CCD1s from various species including tomato, maize, strawberry, lemon, petunia, grape, rice and melon (Bouvier et al., 2003a; García-Limones et al., 2008; Ibdah et al., 2006; Ilg et al., 2009; Kato et al., 2006; Mathieu et al., 2005; Simkin et al., 2004a; Simkin et al., 2004b; Sun et al., 2008). By re-analyzing AtCCD1, LeCCD1 (tomato) and ZmCCD1 (maize), additional cleavage activity of CCD1 was discovered. CCD1 is able to cleave the C5-C6 (C5'-C6') double bonds of lycopene, yielding volatiles including 6, methyl-5-hepten-2-one and pseudoionone (Vogel et al., 2008). This activity was found to be restricted to lycopene (Vogel et al., 2008). Recently, the third cleavage activity of CCD1 subfamily was demonstrated by OsCCD1 *in vitro* and *in vivo* (Ilg et al., 2009). In those studies, recombinant OsCCD1 was able to perform C7-C8 double bond cleavage on certain apolycopenals like C27 apo-10'-lycopenal and C27 β-apo-10'-carotenal; linear carotenoids like lycopene; and monocyclic carotenoids like 3-OH-γ-carotene, leading to the formation of volatile C10 geranial (Ilg et al., 2009). Geranial is an aromatic volatile contributing to the taste of tomato and watermelon (Lewinsohn et al., 2005), as well as the fragrance of rose (Gang,

2005). Although there are three cleavage sites, there were reports showing recombinant OsCCD1 prefers cleavage at C9-C10 (C9'-C10') bond the most, while least at C7-C8 (C7'-C8') and C5-C6 (C5'-C6') double bond. The later only occurs on certain substrates in specific conditions (Ilg et al., 2009; Vogel et al., 2008).

Although enzymatic activities of CCD1 have been studied i*n vitro* extensively, the biological function of these cleavages and the products in plants is unknown. Knockout mutant of CCD1 in *Arabidopsis* by T-DNA insertion resulted in a slight increase of seed carotenoid content, especially for β-carotene (Auldridge et al., 2006), suggesting that AtCCD1 is involved in carotenoid catabolism in seeds. Knockdown mutants of CCD1 in tomato and petunia (*Petunia hybrida*) flowers resulted in significant reduction of C13 aromatic volatiles emission (Simkin et al., 2004a; Simkin et al., 2004b). These volatiles include β-ionone, pseudoionone, and geranylacetone, which are the cleavage products of carotenoids at C9-C10 double bond, and are important components of aromas and flavors in flowers and fruits (Simkin et al., 2004a; Simkin et al., 2004b). This indicates that CCD1 has important role in the production of carotenoids-derived aromatic volatiles in flowers and fruits, which is implicated in pollinator attraction and seed dispersal.

Besides volatiles release, CCD1 also takes part in the production of C13 cyclohexenone and C14 mycorradicin in roots during arbuscular mycorrhizal (AM) fungi colonization. These two apocarotenoids are suggested to be involved in the plant-microbe interaction with not yet clear mechanism (Floss et al., 2008; Schliemann et al., 2008; Sun et al., 2008). When CCD1 expression was silenced in mycorrhizal roots of maize and *Medicago trucncatula*, there were reductions of

these two apocarotenoid accumulations together with an increment of C27 apocarotenoid acid derivative accumulation (Floss et al., 2008; Sun et al., 2008). Relationship between apocarotenoid accumulation with AM fungi symbiosis have been summarized in a recent review (Walter et al., 2010).

Carotenoid Cleavage Dioxygenase 4 (CCD4)

Among the CCD members, least information is available for CCD4. Characterizations of CCD4 are mostly available on formation of petal color, pigments and flavor. CCD4 activity is associated with white petal color formation in chrysanthemum (*Chrysanthemum morifolium* Ramat.). Flowers of chrysanthemum are available in different colors, in which, yellow petal color is derived mainly from carotenoid accumulation (Kishimoto et al., 2004; Ohmiya et al., 2006). White color in petals is due to carotenoid catabolism mediated by the activity of CmCCD4a, whereas the yellow petal variety carried a deletion in this gene. Expression of CmCCD4a is restricted only to petal whereas its isoform, CmCCD4b, is highly expressed in leaves and stems but has almost no expression in petals. Both of these genes have nearly no expression in roots (Kishimoto et al., 2004). In flower petals, both yellow and white cultivars have similar expression levels for all the carotenogenic genes (Kishimoto and Ohmiya, 2006), indicating the lost of pigmentation in white-cultivar is not related to deficiency in carotenoid biosynthesis. PCR-select subtraction screening identified the causative gene is *CmCCD4a* (Ohmiya et al., 2006). Further experiment revealed CmCCD4a is highly expressed in white-cultivar but not in petals of yellow-cultivar. And the expression level of CmCCD4a is correlated with the decrease of carotenoid content in petal during development. When expression of CmCCD4a is knocked down by RNA interference, petal color is changed from white to yellow, which

confirmed the contribution of CmCCD4a to carotenoid catabolism in flower petals, and thus the involvement of CCD4 in carotenoid catabolism (Ohmiya et al., 2006). Although relationship between CmCCD4a and petal color has been established, molecular nature of this enzyme has not been characterized in Ohmiya's study (2006).

Biochemical activity of CCD4 was first reported on CsCCD4a and CsCCD4b isolated from *Crocus sativus*. Both of them showed oxidative cleavage activities at C9-C10 (C9'-C10') positions on β-carotene and produces C13 β-ionone (Rubio et al., 2008). Further experiments were performed on AtCCD4 cloned from *Arabidopisis*, MdCCD4 from apple (*Malus domestica*), CmCCD4a from chrysanthemum (*Chrysanthemum morifolium*), RdCCD4 from rose (*Rosa damascene*) and OfCCD4 from osmanthus (*Osmanthus fragrans*) (Huang et al., 2009). Surprisingly, although all of these CCD4s belong to the same cluster in sequence alignment (Figure 2), they showed differences in substrate preferences (Huang et al., 2009). CmCCD4a and MdCCD4 cleaved C40 β-carotene into β-ionone, but displayed extremely low activity on the C30 8'-apo-β-caroten-8'-al. In contrast, OfCCD4, AtCCD4 and RdCCD4 had almost no detectable activity on β- carotene, instead, they cleaved 8'-apo-β-caroten-8'-al efficiently *in vivo*. It is intriguing that enzymes belonging to the same subclass have different substrate preferences. Additionally, they showed variations in gene structures and expression patterns as well. RdCCD4 and AtCCD4 are intronless while CmCCD4a, MdCCD4 and OfCCD4 contain intron(s) ranged from one to two (Huang et al., 2009; Ohmiya et al., 2006; Tan et al., 2003). NCEDs, enzymes that belong to another subclass of CCD family, are all intronless. It has been proposed that intronless can help to speed up NCED gene expression for ABA biosynthesis

under stress conditions (Tan et al., 2003). However, whether this is true or not in the case of CCD4 is not clear.

Additional cleavage activities for CCD4 at different positions have been reported, but not yet confirmed for two enzymes that clustered into same group as CCD4 in the phylogenetic tree (Figure 2). The first one is BoLCO (lycopene cleavage oxygenase of *Bixa orellana*), an enzyme that cleaves lycopene at C5-C6 (C5'-C6') double bonds, leading to the production of bixin (Bouvier et al., 2003a). It is isolated using RT-PCR by degenerative primers designed based on VP14 sequence. The second one is CsZCO (zeaxanthin cleavage oxygenase of *Crocus sativus*). It is an enzyme responsible for the first step in crocin production by cleaving zeaxanthin at C7-C8 (C7'-C8') double bonds (Bouvier et al., 2003b), and is isolated by RT-PCR using degenerative primers. Primers were designed based on putative sequence of BoLCO. Although these two enzymes are cloned from two different species, they shared a remarkable high identity of 97%. It is uncommon to see conserved proteins from two distantly related species shares such a high identity. On the other hand, the incomplete sequence of CsZCO (369amino acid) is 100% identical to a portion of full length CsCCD4 sequences (580amino acids for CsCCD4a; 589 amino acids for CsCCD4b) (Rubio et al., 2008), together with the absence of any plastid-targeting signal for CsZCO, this suggested *CsZCO* might be a truncated version of *CsCCD4a* or *CsCCD4b*. Combined with the fact that different cleavage activities have been demonstrated by *CsCCD4*s and *CsZCO*, we may have to reconsider the validity of both CsZCO and BoLCD activities. So far, there were no report about CCD4 enzymes that exert the same cleavage activity as that of BoLCO and CsZCO.

Although actual substrate of CCD4 in *planta* has not yet been identified, studies in potato tubers may provide clues. Potato cultivars with yellow tubers showed much higher carotenoid content and lower expression of CCD4 than that in white tubers cultivars. When CCD4 expression is knocked down, increment of violaxanthin and neoxanthin content was observed in the transgenic tubers (Campbell et al., 2010). This suggested the identities of CCD4 substrates in plant *in vivo*. Accompanied with alternations in carotenoid content, heat-stress phenotypes such as dumbbell shaped tubers and microtubers were also reported in the transgenic plants (Campbell et al., 2010). Although without detailed studies, this implicates a novel function of CCD4 in temperature sensing or regulation in heat-stress response in plants.

CCD4 is similar to CCD1 in biochemical activity. All the reported CCD4 orthologues, except BoLCO and CsZCO, cleaved substrates at C9-C10 (C9'-C10') bonds, however, with a much higher enzymatic activity and narrower substrate spectrum than that of CCD1(Huang et al., 2009; Rubio et al., 2008; Schwartz et al., 2001). CCD1 was proven to be closely related to β-ionone emission in flowers and fruits (Simkin et al., 2004a; Simkin et al., 2004b). For CCD4s, all the orthologues studied are expressed in flowers or flower-related organs except CmCCD4b, however, not all of them contribute to β-ionone emission because AtCCD4 and RdCCD4 do not cleave β-carotene well into β-ionone (Huang et al., 2009; Rubio et al., 2008). Moreover, CCD1 does not contain any plastid-targeting transit peptide, and was localized in cytoplasm (Auldridge et al., 2006), whereas CCD4s contain transit peptides and were reported to be localized in plastoglobules (Rubio et al., 2008; Ytterberg et al., 2006), a special vesicle located in plastids and contains lipids and carotenoids. All these suggested that CCD4s

may exert different biological roles in plants when compared to CCD1.

Accumulating evidences as discussed above strongly suggest carotenoid cleavage dioxygenases are (CCD) involved in carotenoid catabolism. Yet, whether or how CCDs play a role in carotenoid accumulation is not known. As human must obtain vitamin A source from the diet and cereals are our staple crops, answering these questions will provide the basis for engineering high provitamin A crops by reducing the degradation mediated by CCDs. This is particularly the case of "Golden rice" where the substrates are enriched via high level of expression of carotenogenic genes (Paine et al., 2005; Ye et al., 2000). In this study, we found that OsCCD1 is the only CCD highly expressed in developing seeds in rice. It encodes a functional CCD1 capable of cleaving a spectrum of carotenoids. To genetically address the role of OsCCD1, we further created transgenic rice knocking down the expression of OsCCD1 in seeds or in the whole plant. We also created transgenic lines of Golden rice with modified promoters to maximize the expression of PSY in conjunction with the RNAi of OsCCD1. This work provides an excellent model for analyzing the role of OsCCD1 in regulating carotenoid accumulation in seeds.

Chapter 3. Hypothesis and objectives

Rice endosperm is carotenoid-free. Owning to the fact that CCDs are able to perform oxidative cleavage activities on a variety of carotenoids, we have the following hypothesis:

(1) Low expression and low activities of carotenogenic enzymes are not the only causative reason for the low carotenoid content in rice seed endosperms.

(2) Members of CCD family are the potential players for carotenoid catabolism in rice seed endosperms

(3) Carotenoid content in rice seed endosperms will increase when expressions of CCDs are knocked down.

To test our hypothesis, the following objectives are set up:

(1) To determine the potential CCD gene(s) responsible for carotenoid turnover in developing rice seeds.

(2) To investigate their involvements in carotenoid catabolism in developing rice seeds

(3) To create high carotenoid transgenic rice seeds by knocking down the potential catabolism.

To reach our objectives, potential candidates responsible for carotenoid turnover in rice seeds will be determined by gene expression studies. Then, candidate CCD genes will be knocked down in rice by RNA interference (RNAi) in endosperm-specific manner. In order to study any possible physiological role of CCDs, candidate genes will also be knocked down in constitutive manner. Carotenoid content in mature transgenic seeds will be analyzed by HPLC.

Chapter 4. Materials and methods

4.1. General cloning and sequencing

Escherichia coli strain (One shot®TOP 10) (Invitrogen, USA) was used in plasmid transformation during the cloning processes. *Agrobacterium tumefaciens* strain EHA105 was used in rice callus transformation. All the primers in PCR cloning, sequencing and probe synthesis were purchased from Invitrogen (USA). DNA sequencing was conducted by Beijing Genomics Institute (Hong Kong) and TechDragon Limited (Hong Kong).

4.2. Extraction of RNA and DNase treatment

Sample to be analyzed was grinded into fine powder with mortar and pertle in liquid nitrogen. Total RNA was extracted using RNeasy® Plant Minikit (QIAGEN) according to manufacturer's protocol. The grinded samples were first lysed and homogenized with buffer containing guanidine hydrochloride and β -Mercaptoethanol. After addition of ethanol, lysate was loaded onto RNeasy silica membrane. RNA in the sample was bind on the membrane, while other contaminants were washed away with ethanol. After that, the pure and concentrated RNA was eluted in RNase free water for later analysis. Concentration of RNA was measured at 260nm using a photometer (Eppendorf). To eliminate DNA contaminations, 10μg of RNA from each sample was resuspended in 1x DNaseI Reaction buffer (New England Bio Labs, Inc.) to a final volume of 100 μl, followed by incubation with 2 units of DNaseI (New England Bio Labs, Inc.) at 37℃ for 10 minutes. Reaction of the enzyme was inactivated by addition of 0.5M EDTA (to a final concentration of 5mM) and

incubation at 75℃ for 10 minutes.

4.3. Reverse transcription

Total RNA was extracted from 100mg of endosperms of different developmental stages as described in section 4.2. After DNase treatment, 4μg of RNA was used as template to synthesize the first strand cDNA by using SuperScript^{TM}III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The cDNA was diluted by 100 times and used to perform RT-PCRs to check the expression pattern of genes involved in carotenoid metabolism. Primers used were designed according to the complete cDNA sequences reported in BLAST database as described in table 2. The PCR reaction mixture included 0.625 unit of polymerase (NEB), 1x standard *Taq* reaction buffer (NEB), 200μM dNTP, 0.2μM forward primer, 0.2μM reverse primer and variable amount of template RNA in a 25μl reaction. The PCR cycles were 94℃ for 2 minutes, followed 30 cycles of denaturation at 94℃ for 15 seconds, annealing at 58℃ for 40 seconds and extension at 72℃ for 1 minute per 1kb, and a final extension at 72℃ for 5 minutes. Standardization of total RNA amount to be used for each sample was carried out by either using total RNA amount or by normalizing the expression level of a constitutive gene, actin.

Table 2. Primers for checking expression profiles of carotenogenic enzymes

Gene names	Name of oligos	Nucleotide sequences
Phytoene synthase 1 (Os06g0729000)	OsPSY1-RTF1	5' **CATGCTTGATGCTGCACTTT** 3'
	OsPSY1-RTR1	5' **CCCTTTATCTCCTCTTCCCG** 3'
Phytoene synthase 2 (Os12g0626400)	OsPSY2-RTF2	5' **CCTGAAAGGCGCAAAGCTG** 3'
	OsPSY2-RTR2	5' **CGATAGCATCAAGGATCTGCC** 3'
Phytoene synthase 3 (Os09g0555500)	OsPSY3-RTF1	5' **CGAGGTACAGGAGCTTCGAC** 3'
	OsPSY3-RTR1	5' **GGGGAGCATGAGTGATCTGT** 3'
Phytoene desaturase (Os03g0184000)	OsPDS-RTF1	5' **GTTGGTGGCCAAGCTTATGT** 3'
	OsPDS-RTR1	5' **GCAGGAGCAAAGACCAACTC** 3'
ξ-carotene desaturase (Os07g0204900)	OsZDS-RTF1	5' **AGCAGATGCATATGTCGCAG** 3'
	OsZDS-RTR1	5' **ATCATTTCCTGGTGACTCGC** 3'
Lycopene β-cyclase (Os02g0190600)	OsLCYb-RTF1	5' **CGACAAGATGCTGTTCATGG** 3'
	OsLCYb-RTR1	5' **AGAACTCCCTCTGCCTCCTC** 3'
Lycopene ε-cyclase (Os01g0581300)	OsLCYε-RTF1	5' **CGAGGAGGAATGGTCCTACA** 3'
	OsLCYε-RTR1	5' **GAAGAAAGCGTCGAACCAAG** 3'
Carotenoid Isomerase (EF417892)	OsCRTISO-F1	5' **CCGTCATGTTCGGCTTCTC** 3'
	OsCRTISO-R1	5' **AAACTGAGGCTTTGACACCC** 3'
Actin (Os10g0510000)	OsActin-1	5' **TGTAAGCAACTGGGATGA** 3'
	OsActin-2	5' **CCTTCGTAGATTGGGACT** 3'

* Nucleotide sequences in **BOLD** indicates coding sequences of the cloned gene

4.4. Real-time quantitative RT-PCR

Total RNA was extracted from 100mg whole seeds of different developmental stages as described in section 4.2. After DNase treatment, TaqMan one-step real-time PCR was carried out using GeneAmp 5700 Sequence Detection (Perkin-Elmer). 25μl PCR reaction mixture contained 1xTaqMan buffer (Perkin-Elmer), 5mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 100nm TaqMan probe, 0.625 units of AmpliTaq Gold polymerase, 0.25 units of multiScribe RNA reverse transcriptase, 200nm gene-specific TaqMan probe, 800nm forward primer, 800nm reverse primer and variable amount (100-300ng) of template RNA. Primers used were designed according to the complete cDNA sequences reported in BLAST database as described in table 3. Condition of PCR was set as the following: 48℃ for 30 minutes, 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 15 seconds, then 60℃ for 1 minute. Standardization of total RNA amount to be used for each sample was carried out by using total RNA amount

Table 3.Primers for checking expression profiles of CCD gene family members

Gene names	Name of oligos	Nucleotide sequences
OsCCD1 (Os12g0640600)	OsCCD1-1	5′ **CCCAACTGCTTCATATTCCAC** 3′
	OsCCD1-2	5′ **TAGCTCAACAACTGCCACC** 3′
OsCCD4a (Os02g0704000)	OsCCD4a-1	5′ **CTCGGTCACTCACTCACTCA** 3′
	OsCCD4a-2	5′ **GGCACGAAGTTGCTGGTCA** 3′
OsCCD4b (Os12g0435200)	OsCCD4b-1	5′ **TGACTTTTGCCCACCCAG** 3′
	OsCCD4b-2	5′ **CACAGGCTCCGACAACAG** 3′
OsCCD7 (Os04g0550600)	OsCCD7-1	5′ **TGTCAAGCTGCTCCTACCA** 3′
	OsCCD7-2	5′ **CAAGTGCATTCTCTGTCCC** 3′
OsCCD8a (Os01g0566500)	OsCCD8a-1	5′ **GCACGGCTCCTAGATCATC** 3′
	OsCCD8a-2	5′ **TGACGAGGCCGACGAGAT** 3′
OsCCD8b (Os01g0746400)	OsCCD8b-1	5′ **CACCATTACAGATAACGGCAC** 3′
	OsCCD8b-2	5′ **TTCCAGGGGTTAGGCAACTC** 3′
Actin (Os10g0510000)	OsActin-1	5′ **TGTAAGCAACTGGGATGA** 3′
	OsActin-2	5′ **CCTTCGTAGATTGGGACT** 3′

* Nucleotide sequences in **BOLD** indicates coding sequences of the cloned gene

4.5. Cloning of OsCCD1 cDNA

Based on EST and genomic sequences deposited in the GenBank (Os12g0640600), a pair of primers was designed (forward "OsCCD1-F1": 5′-ATTCAATCCC CCATACCCAT-3′ and reverse "OsCCD1-R1": 5′-AGCCAATTATTTCCCCGA GT-3′). Predicted *OsCCD1* cDNA was amplified by RT-PCR from *Oryza sativa* L.ssp. *Japonica* (cv. Nipponbare) using high fidelity proof reading DNA polymerase *Pfx* (Invitrogen).

4.6. Bacterial *in vivo* assay of OsCCD1 activity

Predicted coding region of *OsCCD1* cDNA was amplified from leaves of rice [*Oryza sativa* L.ssp. *Japonica* (cv. Nipponbare)] by RT-PCR, forward primer "OsCCD1-EF1" (5′-CACCATGGGAGGCGGCGATGGCGATGAG-3′) and reverse primer "OsCCD1-R1" (5′-AGCCAATTATTTCCCCGAGT-3′) were used. The PCR product was cloned into Gateway® entry vector pENTR-D (Invitrogen) and confirmed by sequencing. *OsCCD1* fragment was then cloned into a Gateway® destination vector, pDEST14 (Invitrogen). To express OsCCD1 protein in bacterial system, the pDEST14 vector harboring our gene fusion construct was transformed into arabinose-inducible *E. coli* strain BL21-AI™ (Invitrogen) that was engineered to accumulate specific carotenoids (courtesy of F. Cunningham, University of Maryland).

To express OsCCD1 protein in bacterial system, the pDEST14 vector harboring our gene fusion construct was transformed into Arabinose-inducible *E. coli* strain BL21-AI™ (Invitrogen) that has been previously transformed with different carotenoid accumulation genes respectively (courtesy of F. Cunningham, University of Maryland).

Individual clones of *E.coli* were inoculated and grown in 100ml LB with 2% glucose at 30℃ until OD_{600} reached about 0.5. Then, 0.2% Arabinose was added to the bacterial culture to induce protein expression, and allowed to grow for additional 8 to 12 hours. Volatile products produced from the culture were collected during and after the induction by blowing air into the head space of the flask that contains the culture. Volatiles were collected by a SuperQ filter trap when out flow air was passed through. Then, volatiles collected were eluted in

hexane, and be concentrated by blowing nitrogen onto the sample. Concentrated samples were analyzed by gas chromatography which was performed by Prof. Andrew J. Simkin (University of Florida, USA).

4.7. Construction of OsCCD1 RNAi constructs

Two *OsCCD1* RNAi cassettes were constructed. One is under the control of the maize constitutive ubiquitin promoter (*Ubi-1*) and the other is under a rice endosperm- specific glutelin-C promoter (*GluC*) respectively. Studies on temporal and spatial expression patterns on rice glutelin promoters in developing seeds indicated that *Glu-C* promoter (Acc. EU264107) conferred high level of expression in both aleurone and starchy endosperm cells (Qu and Takaiwa, 2004; Qu et al., 2008). Hence, Glu-C promoter was chosen to drive the *OsCCD1*-RNAi expression.

Structure of RNAi cassettes, namely "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1-RNAi" was shown in figure 4a and 4b, in which construction of "OsCCD1-Ubi" was completed by Dr. Duan, Yuanlin (Fujian Agriculture & Forestry University, China). To start with, an *OsCCD1* fragment and *GluC* promoter were amplified from rice genomic DNA [*Oryza sativa* L.ssp. *Japonica* (cv. Nipponbare)] by PCR using gene specific primers (listed in table 4). Then, the fragments were ligated into pCR4-TOPO vector (Invitrogen). After sequencing to confirm the sequence fidelity, *OsCCD1* fragments were retrieved from the vector by restriction enzyme digestion. Through multiple steps of restriction enzyme digestions and ligations (refer to figure 5a and 5b), *OsCCD1* RNAi cassettes were inserted into binary vector pTCK303. pTCK303 is a PCR based RNAi vector, which contains a maize constitutive *Ubi-1* promoter and a rice intron flanked by

two multiple cloning sites (Wang et al., 2004).

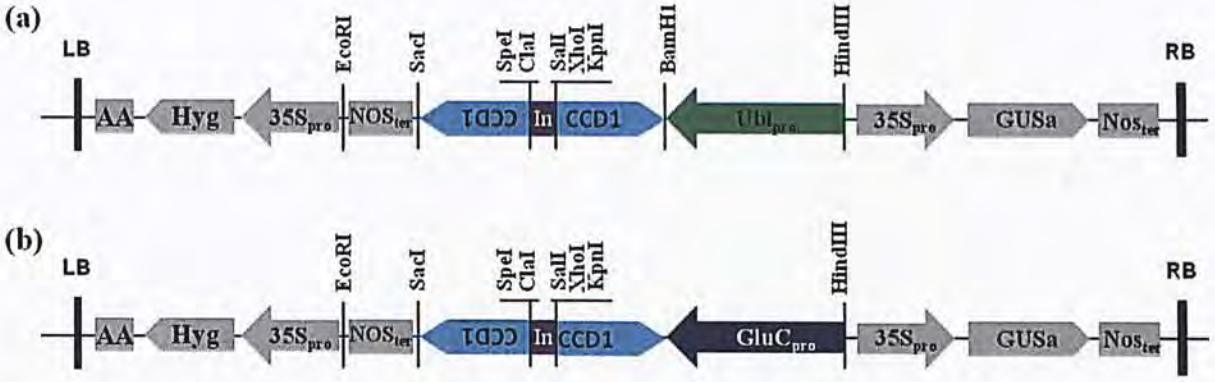

Figure 4. Structures of two RNAi cassettes in pTCK303 used in rice callus transformation. (a) *OsCCD1* RNAi cassette driven by a *Zea mays Ubi-1* promoter, named as "Ubi-OsCCD1-RNAi". (b) *OsCCD1* RNAi cassette driven by *GluC* promoter (GenBank accession number: EU264107), named as "GluC-OsCCD1-RNAi". Purple rectangles, rice intron; LB, left border; RB, right border; Pro, promoter; Ter, terminator; AA, 35S polyadenylation signals; In, rice intron; GUSa,, β-glucuronidase reporter gene; Ubi$_{pro}$, *Zea mays Ubi-1* promoter; GluC$_{pro}$, GluC promoter.

Table 4. Primers for PCR cloning in making OsCCD1 RNAi constructs

Gene name	Name of oligos	Restriction sites	Nucleotide sequences
Glu-C promoter (EU264107)	Gt3-H3-PF1	*Hin*dIII	5′ GGAAGCTT**GTTCAAGATTTATTTTTGG** 3′
	Gt3-Bg2-PR1	*Bgl*II	5′ GCAGATCT**AGTTATTCACTTAGTTTCCC** 3′
OsCCD1 (Os12g0640600)	OsCCD1-KpnI	*Kpn*I	5′ GGGGTACC**CCCAACTGCTTCATATTCCAC** 3′
	OsCCD1-BamHI	*Bam*HI	5′ CGGGATCC**TAGCTCAACAACTGCCACC** 3′
	OsCCD1-SpeI	*Spe*I	5′ GGACTAGT**CCCAACTGCTTCATATTCCAC** 3′
	OsCCD1-SacI	*Sac*I	5′ CGGAGCTC**TAGCTCAACAACTGCCACC** 3′

* Nucleotide sequences in **BOLD** indicates coding sequences of the cloned gene; UNDERLINED indicates restriction enzyme sites to be conferred on the cloned fragments.

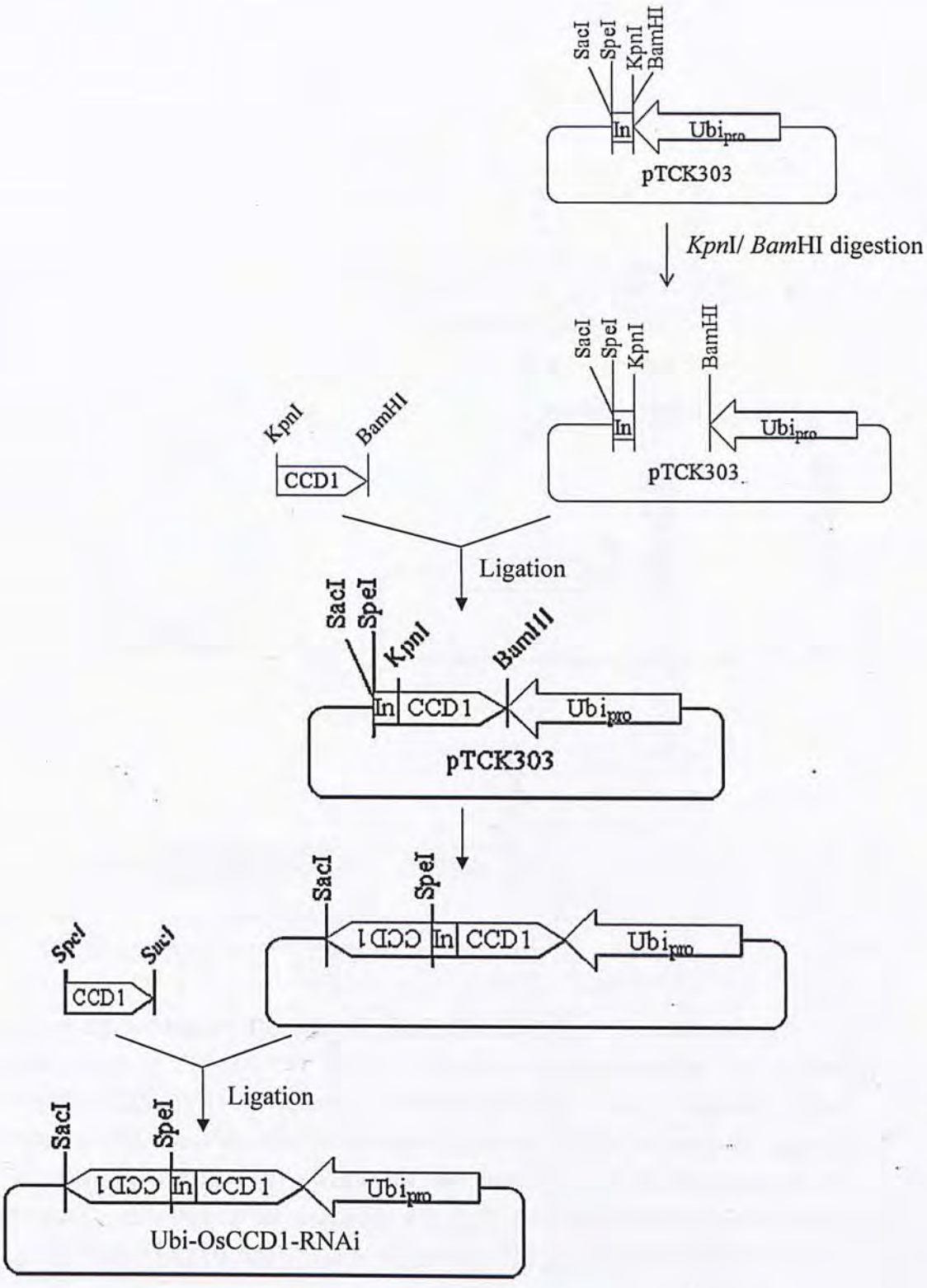

Figure 5a. Schematic diagram of "Ubi-OsCCD1-RNAi" construction.

Binary vector pTCK303 was cut open by *Kpn*I and *Bam*HI digestion. Then, *CCD1* fragment was ligated into the vector. The resulted plasmid was cut open by *Sac*I and *Spe*I, followed by insertion of *CCD1*.

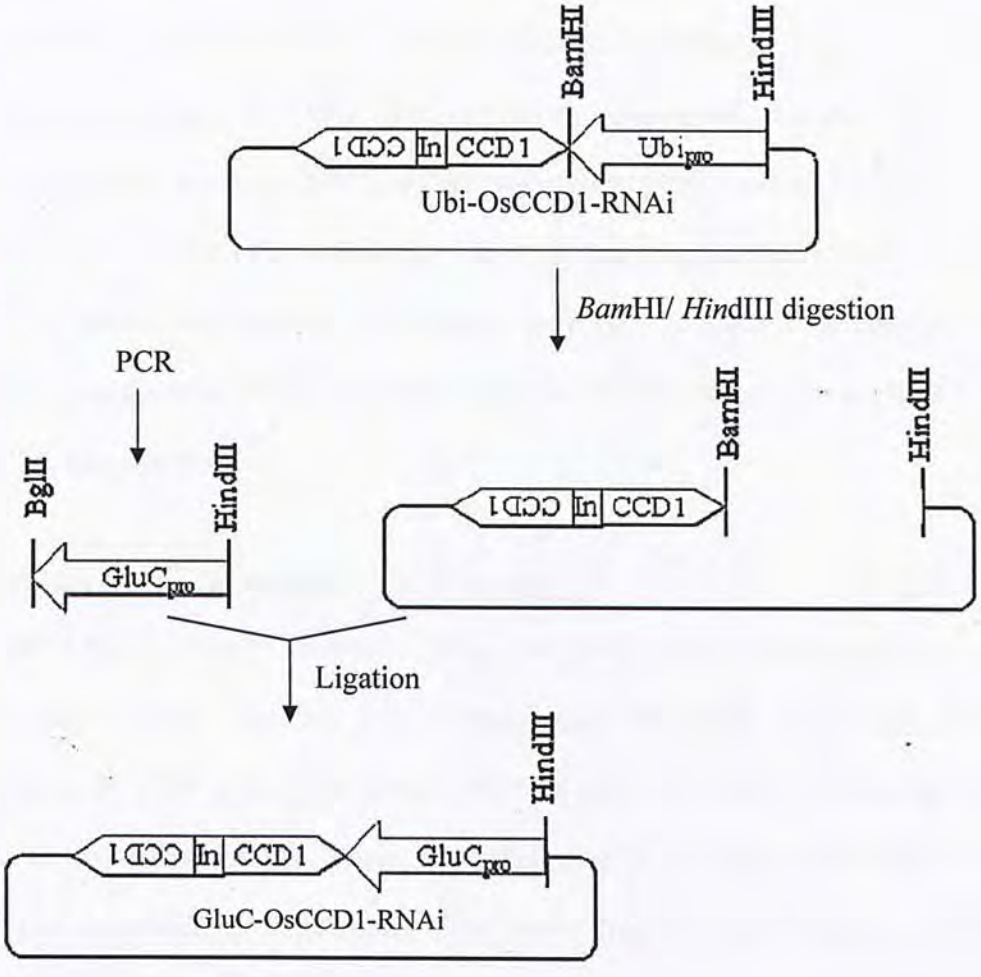

Figure 5b. Schematic diagram of "GluC-OsCCD1-RNAi" construction.

The construct "Ubi-OsCCD1-RNAi" was used as the backbone for making "GluC-OsCCD1-RNAi" cassette. *Ubi-1* promoter was removed from "Ubi-OsCCD1-RNAi" by *Bam*HI and *Hin*dIII digestion. Then, *GluC* promoter fragment was cloned by PCR and *Bgl*II/ *Hin*dIII sites were engineered at 5' and 3' end respectively. As sticky ends of *Bam*HI are compatible with *Bgl*II, *GluC* promoter was ligated into the opened "pUbi-OsCCD1-RNAi" vector that had its *Ubi*-1 promoter removed.

4.8. Construction of "Super-Golden" rice constructs

Two transgenic constructs namely "pGT-PCC" and "pGYGC" were designed as shown in figure 6a and 6b. To facilitate the construction, two cassettes namely "GluC-Y1-Nos" and "Gt1-TCN" were constructed beforehand. "pGT-PCC" construct is built based on "GluC-OsCCD1-RNAi" (figure 4b) construct with insertions of gene cassettes over-expressing two carotenogenic biosynthetic gene, *Psy* and *CrtI*. As *CrtI* is of bacterial origin, a plastid-targeting signal peptide from RBS small subunit was fused to *CrtI* to target the protein to plastids. Designs of these two gene cassettes "GluC-Y1-NosT" and "Gt1-TCN" were shown in figure 7a and 7b, respectively.

4.8.1. Construction of "GluC-Y1-Nos" cassette

To build "GluC-Y1-NosT" cassette, $GluC_{pro}$ and *ZmY1* were cloned from rice [Oryza sativa L.ssp. Japonica (cv. Nipponbare)] and maize (*Zea mays*), respectively, by PCR using gene-specific primers (table 5). Nos_{ter} was derived from a binary vector pBI121. These fragments were ligated into pCR4-TOPO vector and sequenced for verification. Then, *ZmY1* fragment was retrieved and ligated into pCR4-TOPO vector containing $GluC_{pro}$. After sequencing, GluC-Y1 cassette was retrieved from the vector by *Pst*I digestion, and then ligated into pCR4-TOPO vector containing Nos_{ter}. For details, please refer to figure 8a.

4.8.2. Construction of "Gt1-TCN" cassette

To build "Gt1-TCN" cassette, *CrtI* and Nos_{ter} were cloned from pCa and pBI121, respectively by PCR using gene specific primers (table 5). These two fragments were ligated into pCR4-TOPO for sequencing. After verify the sequences, *CrtI* fragment was retrieved from the vector and ligated into *Sph*I/*Sac*I digested pRBS. Nos_{ter} was retrieved from pCR4-TOPO by *Sac*I/*Eco*RI digestion, followed

by ligation into pRBS vector that had been inserted by *CrtI* fragment. Fusion cassette "CrtI-Nos_{ter}" was excised from the vector by *Sph*I/*Eco*RI digestion and ligated into pGEM-3Z vector that had been inserted with *TP*. By *Hin*dIII/*Eco*RI digestion, fusion cassette "TP-CRTI-Nos_{ter}" (*Hin*dIII/*Eco*RI) was isolated, and then ligated into pBluescript KS so as to obtain *Spe*I site at 3' end. Through *Hin*dIII/*Spe*I digestion, fusion cassette "TP-CRTI-Nos_{ter}"(*Hin*dIII/*Spe*I) was retrieved and then ligated into pCR4-TOPO containing *Gt1* promoter. pCa (vector containing *CrtI*) and pRBS (pSP6T3 containing *TP*) used in PCR cloning were provided from my supervisor. For details in building this "Gt1-TCN" cassette, please refer to figure 8b.

4.8.3. Construction of "pGT-PCC"

"GluC-Y1-NosT" and "Gt1-TCN" were inserted into "GluC-OsCCD1-RNAi" (figure 4b) in pTCK303 vector sequentially through multiple steps of digestion and ligation (refer to figure 9).

4.8.4. Construction of "pGYGC"

"pGT-PCC" (figure 6b) construct is built based on pTCK303 (Wang et al., 2004). To do so, 3' end of "GluC-Y1-Nos" cassette was conferred from *Hin*dIII into *Eco*RI by PCR using gene specific primers (table5), followed by insertion into *Hin*dIII/*Eco*RI digested pTCK303 with *Ubi-1* promoter removed. Then, "Gt1-TCN" cassette was inserted into *Eco*RI digested pTCK303 that has been inserted with "GluC-Y1-Nos"cassette. Details of construction scheme were shown in figure 10.

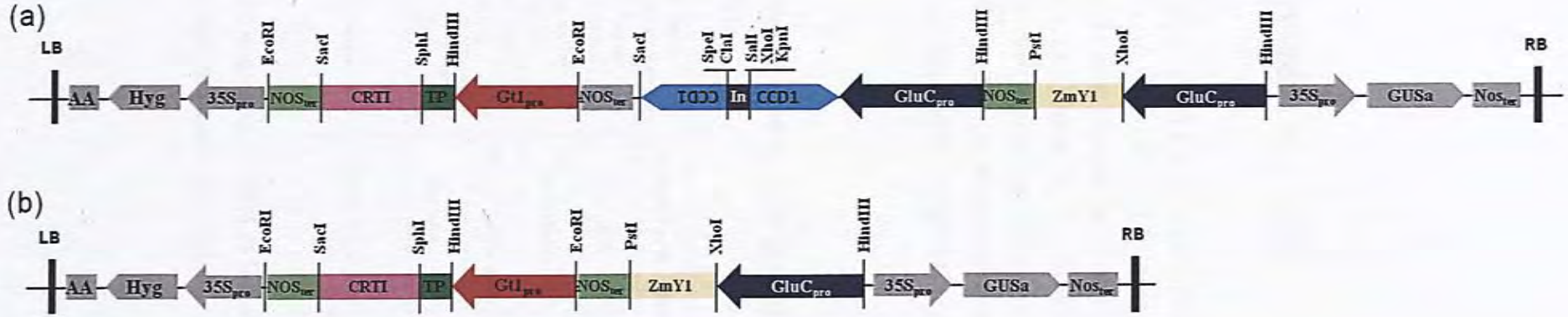

Figure 6. Structures of two super-golden rice RNAi cassettes

(a)Construct with *OsCCD1* RNAi, named as "pGT-PCC".(b)Construct without *OsCCD1* RNAi, named as "pGYGC". Purple rectangles, rice intron; LB, left border; RB, right border; Pro, promoter; Ter, terminator; AA, 35S polyadenylation signals; In, rice intron; GUSa,, β-glucuronidase reporter gene; $Gt1_{pro}$, *Glu4-2* promoter (GenBank accession number: EU264103); $GluC_{pro}$, *GluC* promoter (GenBank accession number: EU264107); CRTI, phytoene desaturase cloned from *Pantoea agglomeran* (GenBank accession number: M38423); TP, transit peptide cloned from pea ribulose bisphosphate carboxylase small subunit (GenBank accession number: X04334); ZmY1, phytoene synthase1 cloned from *Zea mays* (GenBank accession number: AY455286); *NOS* terminator cloned from pBI121 binary vector.

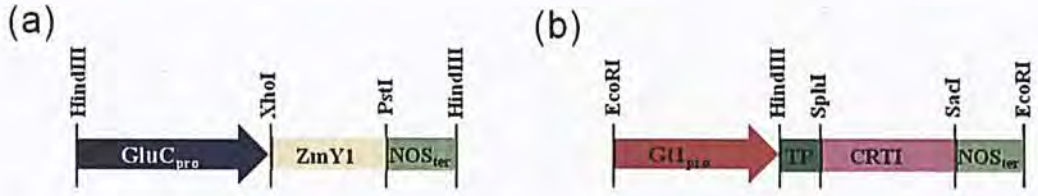

Figure 7. Structure of two fusion cassettes used to build super-golden rice constructs. (a) "GluC-Y1-NosT" consists of glutelin-C promoter (*Glu-C*) (Acc: EU264107), *Zea mays* phytoene synthase 1(*ZmY1*) (Acc: AY455286) and *NOS* terminator from pBI121 binary vector. (b) "Gt1-TCN" consists of Gt1 promoter (Acc: EU264103); transit peptide (*TP*) from *Pisum sativum* ribulose bisphosphate carboxylase small subunit (Acc: X04334.1); phytoene desaturase (*CrtI*) from *Pantoea agglomeran* (Acc: M38423) and *NOS* terminator cloned from pBI121 binary vector.

Table 5. Primers used in PCR cloning for "GluC-Y1-NosT" and "Gt1-TCN" constructions

Gene names (GenBank accession number)	Origin of gene	Sites conferred	Nucleotide sequences
GluA-2 promoter (EU264103)	*Oryza sativa*	5'EcoRI	5′ GGGAATTCCACCCTCAATATTTGGAAAC 3′
		3'HindIII	5′ GGAAGCTTGGATCCGTTGTTGTAGGACT 3′
Glu-C promoter (EU264107)	*Oryza sativa*	5'HindIII	5′ GGAAGCTTGTTCAAGATTTATTTTTGG 3′
		3'XhoI	5′ GCCTCGAGAGTTATTCACTTAGTTTCCC 3′
Carotene desaturase, *crtI* (M38423)	*Pantoea agglomeran*	5'SphI	5′ AGCATGCGGATGAAAAAAACCGTTGTG 3′
		3'SacI	5′ AGAGCTCGTTGGCTCATTGCAGATCCT 3′
Phytoene synthase 1, *psy1* (AY455286)	*Zea mays*	5'XhoI	5′ CTCGAGCCACCACCACCACTCTTCTT 3′
		3'PstI	5′ GGCTGCAGTGTTTTTCATCAAGGCCTCC 3′
NOS terminator	pBI121 vector	5'SacI	5′ GGGAGCTCGATCGTTCAAACATTTGGCA 3′
		3'EcoRI	5′ GGGAATTCGATCTAGTAACATAGATGAC 3′
		5'PstI	5′ GGCTGCAGGATCGTTCAAACATTTGGCA 3′
		3'HindIII	5′ GGAAGCTTGATCTAGTAACATAGATGAC 3′

* Nucleotide sequences in **BOLD** indicates coding sequences of the cloned gene; UNDERLINED indicates restriction enzyme sites to be conferred on the cloned fragments.

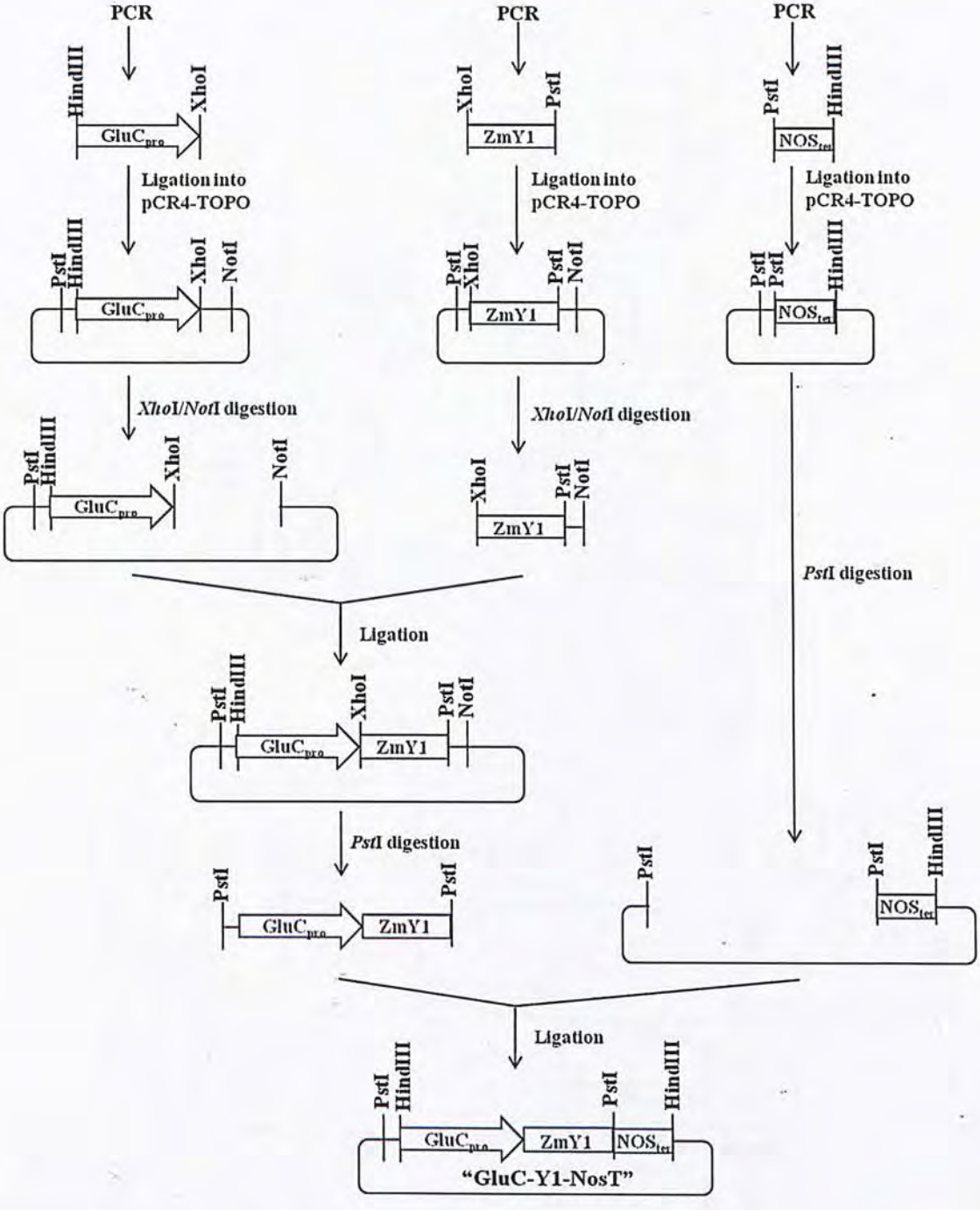

Figure 8a. Schematic diagram of "GluC-Y1-NosT" cassette construction.

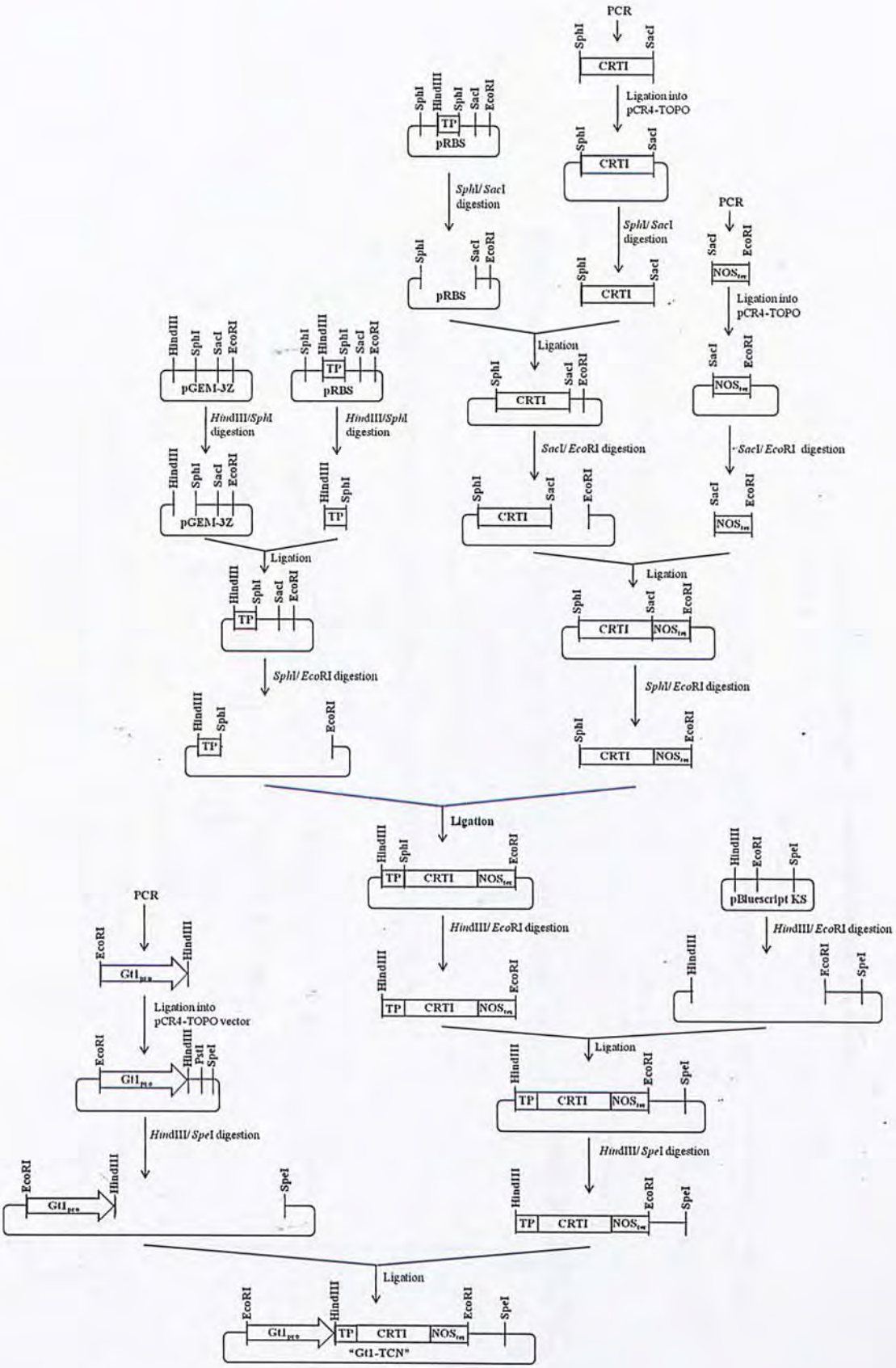

Figure 8b. Schematic diagram of "Gt1-TCN" cassette construction.

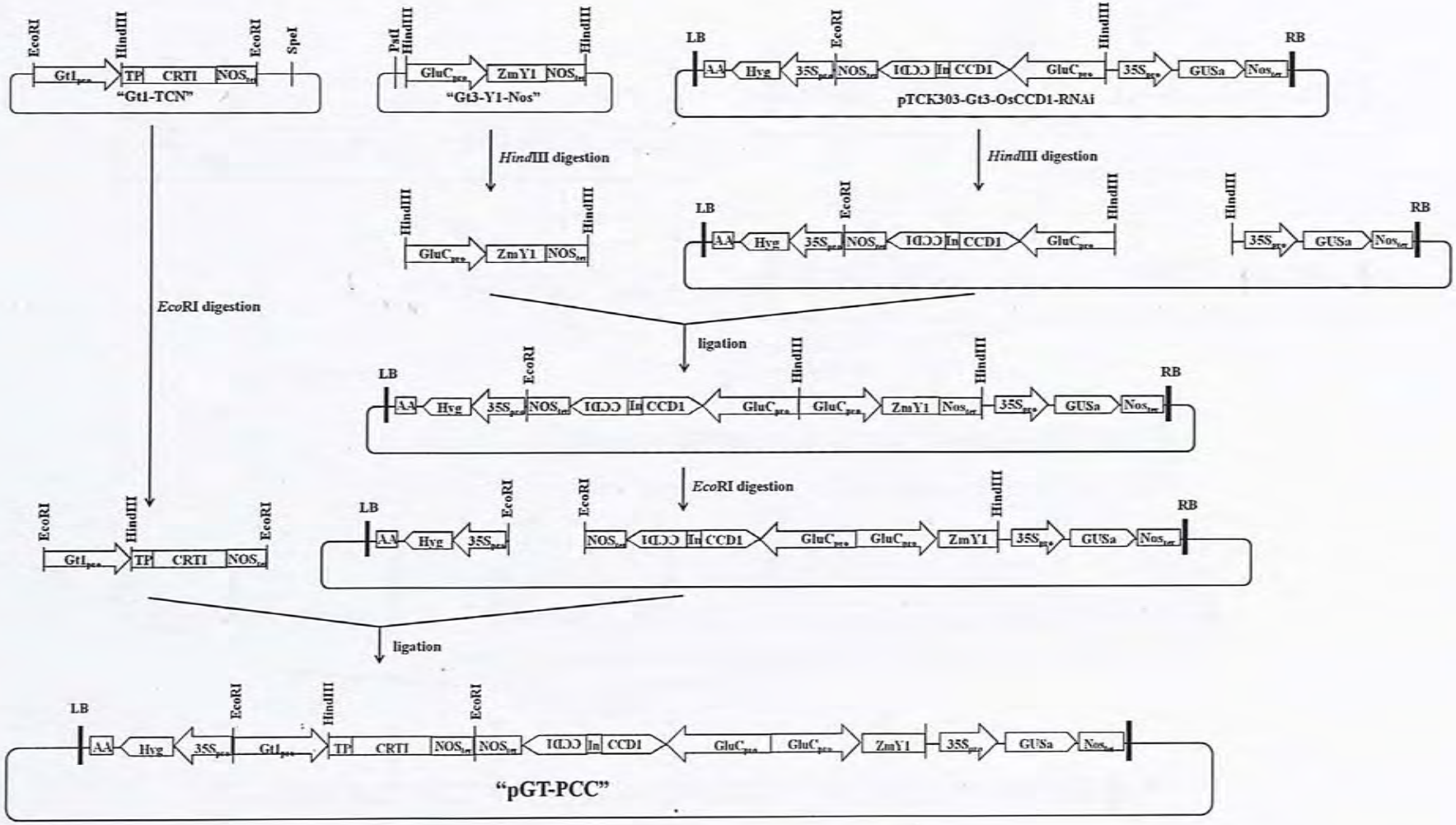

Figure 9. Schematic diagram of "pGT-PCC" construction

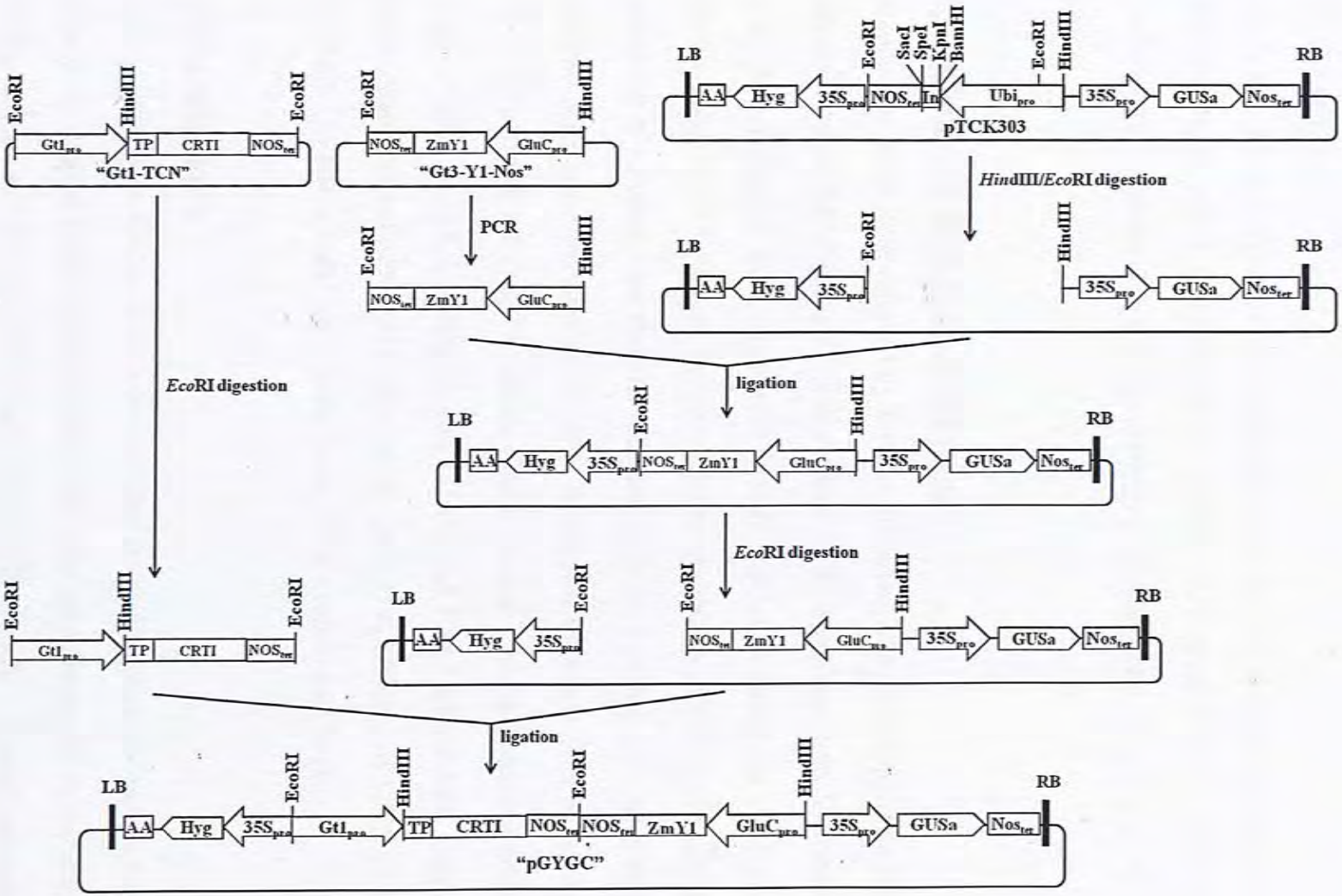

Figure 10. Schematic diagram of "pGYGC" construction

4.9. Rice transformation

Plant materials

Oryza sativa L.ssp. *Japonica* (cv. Nipponbare) was used as the plant material for *Agrobacteria*-mediated transformation. Its seeds were donated as a gift from Professor Yu Weichang (The Chinese University of Hong Kong, Hong Kong SAR, China).

Callus induction from mature rice seeds

Mature rice seeds were dehulled by forceps, sterilized with 70% ethanol for one minute, then washed thoroughly with sterilized Elix water one time. The seeds were then sterilized with commercial bleach (sodium hypochlorite 1%) that contain one drop of Tween-20, by subjecting to continuous shaking on an orbital shaker for 30 minutes. After that, the bleached seeds were washed thoroughly with sterilized Elix water for three times. After blot drying on sterile filter paper, the seeds were transferred onto 2N6 media [30g/L Sucrose , 0.3g/L Casamino acids, 0.5g/L Proline, 0.5g/L L.Glutamin, 4.0g/L CHU(N_6) Basal salt mixture, 2mg/L 2,4-Dichlorophenoxy acetic acid, 3g/L Gelrite, pH5.8] at a density of 12-15 seeds per plate. The plates were kept in darkness at 28℃ for two to four weeks.

Callus subculture

Calli induced from mature seeds were separated from endosperm and radicle with a sterilized surgical blade. After isolation, calli were subcultured into pieces sized around 2.5mm, and transferred to freshly prepared 2N6 media for proliferation by keeping in dark at 28℃ five days before transformation.

Preparation of *Agrobacterium tumefaciens*

Freeze-thaw method was applied to introduce desired plasmid DNA into *Agrobacterium tumefaciens* (strain: EHA105). After confirming the identity of clones by restriction enzyme digestions, the bacteria were streak out on YEP plates (35g/L LB agar, 5g/L Yeast extract, 50mg/L Kanamycin, 50mg/L Rifampicin, 200μg/L Acetosyringone) one day prior to the operation of rice callus transformation.

Co-cultivation

Agrobacteria were scraped off the YEP plate surface and resuspended in liquid 2N6-AS medium [30g/L Sucroce, 0.3g/L Casamino acid, 10g/L Glucose, 4.0g/L CHU(N_6) Basal salt mixture, 10ml/L N6 vitamins(100x), 2mg/L 2,4-Dichlorophenoxy acetic acid, pH5.8] that contain 100μm acetosyringone. *Agrobacteria* were resuspended into OD_{600} about 0.1, and left to sit at room temperature for two to three hours. After that, suspension mixture was transferred into a larger flask. Calli were added, swirled gently and left to sit at room temperature for about 40 minutes. After incubation, excess *Agrobacteria* were removed from the surface of calli by blot drying on sterile filter paper, then individual calli were transferred onto solid 2N6-AS medium [30g/L Sucroce, 0.3g/L Casamino acid, 10g/L Glucose, 4.0g/L CHU(N_6) Basal salt mixture, 10ml/L N6 vitamins(100x), 2mg/L 2,4-Dichlorophenoxy acetic acid, 3g/L Gelrite, pH5.8] that contain 100μm acetosyringone. Rice calli were co-cultivated with the *Agrobacteria* in darkness at 22℃ for two to three days.

Callus washing and selection

After co-cultivation, calli were removed from the plate and transfered to a flask containing sterilized Elix water. Calli were washed by shaking gently on an orbital shaker for 20 to 30 minutes. Washing process was repeated twice with fresh sterilized Elix water.

Calli were blot dried on sterile filter paper and placed onto solid 2N6-TCH medium (30g/L Sucrose, 0.3g/L Casamino acid, 4.0g/L CHU(N_6) Basal salt mixture, 10ml/L N6 vitamins(100x), 2mg/L 2,4-Dichlorophenoxy acetic acid, 3g/L Gelrite, pH5.8) which contain 25mg/L Hygromycin B (for selection of calli transformed with desired plasmid), and 400mg/L Timentin (for killing excess *Agrobacteria*). These were used to select resistant calli at 28℃ for two weeks in darkness. After that, the calli were transferred onto solid 2N6-TCH medium that contain 50mg/L Hygromycin B and 200mg/L Timentin. To allow proliferation, calli were kept in darkness at 28℃ for another two weeks.

Regeneration

After two weeks or beyond, proliferated calli were transferred onto solid regeneration medium, RGH6 [30g/L Sucrose, 0.5g/L L.Glutamine, 0.5g/L Proline, 0.3g/L Casamino acid, 4.0g/L CHU(N_6) Basal salt mixture, 10ml/L N6 vitamins(100x), 3mg/L 6-Benzylaminopurine, 0.5mg/L Naphthalene acetic acid, 50mg/L Hygromycin, 3g/L Gelrite, pH5.8] then incubated in darkness at 28℃ for one week. After that, the calli were transferred to light at 28℃ for one to two weeks. After part of the calli changed from creamy yellow into green, which indicates there were differentiation of chloroplast, those calli were transferred to rooting medium [30g/L Sucrose, 4.0g/L CHU(N_6) Basal salt mixture, 5ml/L N6

vitamins(100x), 50mg/L Hygromycin, 3g/L Gelrite, pH5.8] and incubated at 28℃ in 16 hours/ 8 hours light dark cycle for two weeks. After differentiation of shoots and roots, the transgenic plantlets could then be screened and planted into soil for growth.

Screening of transgenic plants

To screen for transgenic plants, small portion of root (~3mm long) was removed from the plant, and histochemcial staining was performed using β-Glucuronidase Reporter Gene Staining Kit (Sigma) according to the manufacturer's protocol. For successful transformants, their roots would change from creamy white to blue.

4.10. Detection of transgene

4.10.1. Southern blot

Isolation of genomic DNA

1 gram of fresh leaf was harvested from each individual line of transgenic plants. The leaf was grinded into fine powder by mortar and pertle in liquid nitrogen, followed by addition of 5ml DNA extraction buffer [For making 400ml solution: 168g Urea, 25ml 5M Sodium chloride, 20ml 1M Tris-HCl (pH8.0), 16ml 0.5M EDTA (pH8.0), 20ml 20% Sarkosyl, water was added to make total volume into 400ml]. The mixture was allowed to thaw at room temperature with periodic mixing. After that, mixture was transferred to a 12ml centrifuge tube, followed by addition of 4ml phenol:chloroform(1:1, vol/vol). Mixing was shook at 30 rpm on a rocking platform (Bio-Rad) for at least 30 minutes. The tube was then centrifuged at 4800rpm ,10℃, for 20 minutes. Upper layer supernatant was transferred to a new 12ml centrifuge tube, and mixed with 0.1X volume of 3M sodium acetate (pH5.2). Color of the mixture would change from yellow to pale

pink at this time. Then, 3.8ml isopropanol was added, thread-like DNA would appear by inverting the tube five to six times. For better quality of DNA, the tube was shook on a rocking platform at 30 rpm for 5 minutes. DNA pellet was then recovered after centrifuge at 4800rpm,10℃, for 15 minutes. DNA pellet was washed with 1ml 70% ethanol, transferred to a 1.5ml microcentifuge tube and washed with 0.5ml 70% ethanol again. After incubation for 2 to 5 minutes at room temperature, DNA pellet was recovered again by centrifuging at 14,000 rpm for 2 minutes. Supernatant was removed, and the DNA pellet was completely dried under vacuum. To dissolve the pellet, 500μl 1X TE buffer (10mM Tris, 1mM EDTA, pH8.0) was added followed by incubation on ice at 4℃ overnight. The dissolved DNA could be stored in -20℃ until usage.

Southern blot

A 662bp fragment of Hygromycin B resistance gene was used as the probe for southern blot. It was amplified from pTCK303 using forward primer (5′-ATTTCGGCTCCAACAATGTC-3′) and reverse primer (5′-AATTAATT CGGGGGATCTGG-3'). After cloning into pCR2.1 (Invitrogen), it was confirmed by sequencing. DNA fragment was retrieved from the vector by *Eco*RI digestion. To synthesize probes, 45μl of TE buffer that contain about 50ng DNA fragment was incubated in boiling water for 5 minutes, and transferred to ice for 2 minutes immediately afterwards. Condensation was brought down by brief centrifugation. All the solution in the tube was transferred to a tube containing Ready-To-Go DNA Labelling Beads (-dCTP)(GE Healthcare). Beads were completely dissolved, followed by the addition of 5μl [α-^{32}P]-dCTP (3000 Ci/mmol, GE Healthcare). This reaction mixture was incubated at 37℃ for 30 to 60 minutes. Probe mixture was loaded into ProbeQuant™ G-50 Micro Column (GE Healthcare), and

centrifuged at 3,000rpm (750xg) for 2 minutes. The probe was denatured by incubation in boiling water for 10 minutes and then cooled down on ice immediately for 2 minutes.

About 13μg genomic DNA was digested with *Hin*dIII for 6 hours or overnight at 37℃ with the presence of RNase. Digested DNA was precipitated with ethanol and sodium acetate. Residual salts were washed away by 70% ethanol. Pure DNA was dried under vacuum then dissolved in 30μl loading buffer, followed by separation on EB containing 0.7% agarose/TBE gel overnight. DNA fragments were transferred to Hybond™-N membrane (GE Healthcare) by capillary action, and stabilized by UV crosslink. After pre-hybridization, denatured P32-labelled probe was hybridized to the membrane overnight by incubation at 65℃. The membrane was washed with washing buffer [0.1% SDS, 40 mM Na^+ .PO_4 (pH 7.2), 1mM EDTA], then blotted to damp dry prior to X-ray film exposure for detection.

4.10.2. HPLC analysis of carotenoids in seeds

Carotenoid extraction

Extraction protocol was modified from a publication (Sérino et al., 2009). All processes were carried out under low light conditions. 1 g of dehusked T_2 rice seeds were weighted and grinded into fine powder using a coffee grinder (Model: CG100, Kenwood), furthering grinding was achieved by using a mortar and pertle. 0.5 g of powder was transferred to a 2ml micro-centrifuge tube. To estimate the recovery rate of carotenoid extraction, a known amount of Astaxanthin (Dr. Ehrenstorfer, German) was added to the sample before organic extraction. Rice powder was rehydrated by addition of 100μl saturated sodium chloride aqueous

solution and 50μl n-hexane, after agitation on a vortex for 30 seconds at room temperature, centrifugation was performed at 16,873xg for 2 minutes at 4℃. After that, the sample was extracted with 200μl acetone [contain 1% butylated hydroxytoluene(Sigma)] once, 1ml ethyl acetate [contain 1% butylated hydroxytoluene(Sigma)] four times by agitation on a vortex for 30 seconds at room temperature, followed by centrifugation at 16,873xg for 2 minutes at 4℃. Upper layer of supernatant was transferred to a new micro-centrifuge tube, in which the organic solvent was evaporated under vacuum for 1 hour in room temperature, and residue was redissolved in 150μl ethyl acetate. After filtering through a Acrodisc® 13mm syringe filter (0.2μm, PVDF, Aglient), 100μl was injected into HPLC machine for analysis.

HPLC analysis

HPLC machine (model 2996, WATERS, USA) with photodiode array detector (PDA2996, WATERS, USA) was applied in the assay. Separation of carotenoids was achieved using C_{30} reversed-phase YMC carotenoid column (4.6x 250mm, 5μm, WATERS, USA). 20μl aliquots of total carotenoid extracts was injected into the machine immediately after extraction, mobile phase used was developed into linear gradient system with solvent A (methanol: H2O: TBME, 70:25:5, vol/vol) and solvent B (methanol: H2O: TBME, 7:3:90, vol/vol) at flow rate 1ml per minutes. Conditions were set up according to the description in Paine's paper (Paine et al., 2005). Identities of carotenoids eluted out of the column were confirmed by comparing the retention times with that of the HPLC standards purchased from various companies. These include alpha-carotene (Wato, Japan) and beta-carotene (Sigma, USA). For each of carotenoids to be measured, at least five different known concentrations of them were injected into the HPLC machine

using the conditions mentioned as above. Calibration curves of each carotenoid to be measured were then plotted with peak area against concentrations. By comparing peak areas between samples and calibration curves, quantitative analysis of each type of carotenoids could be achieved.

Chapter 5. Results

5.1. Expression profiles of carotenogenic genes in rice endosperms

The low accumulation of carotenoids in rice seeds can be resulted from low carotenoid biosynthesis. To analyze activity of the pathway, mRNA levels of carotenogenic genes were determined by RT-PCR in wild type rice seeds [*Oryza sativa* L.ssp. *Japonica* (cv. Nipponbare)] at different days after flowering (DAF). These genes include *PSY1*, *PSY2*, *PSY3*, *ZDS*, *PDS*, *CRTISO*, *LCYβ* and *LCYε* (refer to Figure 1). Hulls were removed manually by forceps. After removal of the embryo, endosperms were classified into different developmental stages (S2, S3, S4, S5 and S6) based on degrees of maturity (refers to Figure 11). Total RNA was extracted with RNeasy® Plant Minikit (QIAGEN). Rice actin gene was used as a control for equal template. Results of RT-PCRs indicated that all the carotenogenic genes were expressed in different developmental stages albeit levels of some genes were drastically low (Figure 12). It was noted that detection of *PSY1*, *PSY2* and *PSY3* genes required 35 cycles in PCR. In contrast, only 25 to 30 cycles were sufficient to amplify other genes. Extra amplification cycles suggested the low abundances of *PSY1*, *PSY2* and *PSY3* mRNA in developing rice endosperm. This result indicated that the carotenoid biosynthetic pathway is intact and the genes are expressed. The rice seeds may have limited biosynthetic activity because of low expression of *PSY*. PSYs catalyze the rate-limiting step in the carotenoid biosynthetic pathway.

Figure 11. Developing rice seed at different stages, with embryo removed.
S2 (watery stage): Collected at around 3 days after flowering (DAF). Caryopsis was small in size, filling inside was still watery and colorless; **S3 (milky stage):** Collected at around 5DAFs. Caryopsis increased in size and fillings started to change from colorless to milky, but still wet and soft; **S4 (drying stage):** Collected at around 10DAFs. Caryopsis increased rapidly in size, and fillings started to dry up and hardened; **S5 (yellowing stage):** Collected at around 15DAFs. Yellowing of panicle was observed, and caryopsis has reached its maximum size. **S6 (mature stage):** Collected around 20DAFs. The whole grain was fully developed and matured, completely dried and turned into yellow.

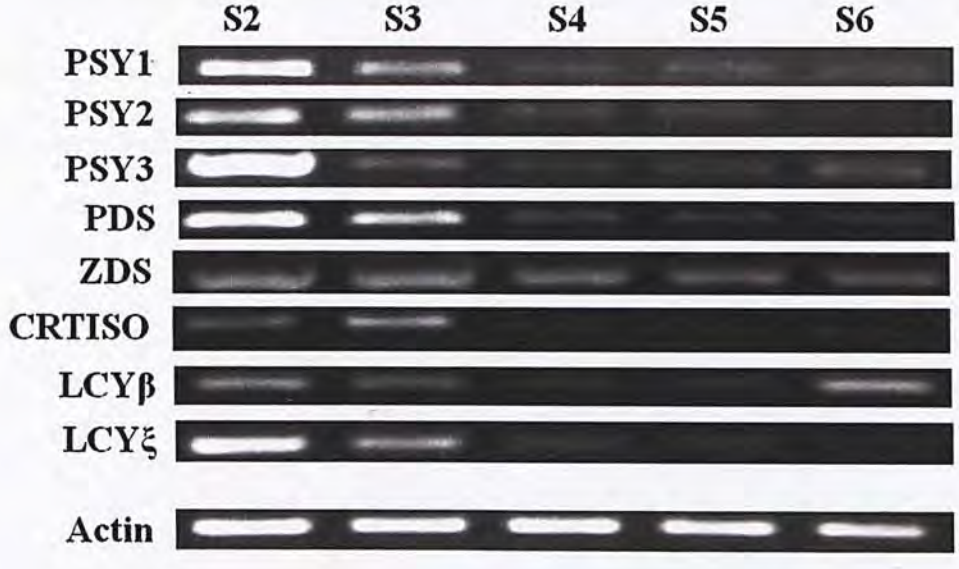

Figure 12. RT-PCR analysis of carotenoid biosynthesis related genes in developing rice endosperm. Detailed description of S2-S6 refers to figure 11. **Actin,** rice actin; **PSY1**, phytoene synthase 1; **PSY2**, phytoene synthase 2; **PSY3**, phytoene synthase3; **PDS**, Phytoene desaturase; **ZDS**, ξ-carotene desaturase; **LCYβ**, Lycopene β-cyclase; **LCYε**, Lycopene ε-cyclase; **CRTISO**, Carotene Isomerase;

5.2. Expression of *CCDs in* developing rice seeds

Increasing evidence suggested that one or more members of CCD subfamily may be involved in carotenoid catabolism in rice seed endosperm. These include increment of carotenoid level in *CCD1* T-DNA knock down *Arabidopsis* seeds (Auldridge et al. 2006), as well as the discovery of *ZmCCD1* function. *ZmCCD1* is encoded by white cap locus (Wc), which is responsible for carotenoid breakdown in maize endosperm (Tan, unpublished data). CCDs belong to a small gene family in plants. To analyze this family, BLAST search was performed with sequences of VP14/NCEDs in maize and *Arabidopsis*. Phylogenetic analysis by ClustalW2 (http://align.genome.jp) indicated that subfamilies of NCED and CCDs are highly conserved among these species (figure 13). The NCED subfamily responsible for ABA biosynthesis is nicely clustered together. CCD7 and CCD8 that are involved in strigolactone biosynthesis were clustered in two branches. CCD1 and CCD4 were in other two branches. Based on this analysis, rice contains a single OsCCD1 and OsCCD7, two OsCCD4 (a,b) and OsCCD8 (a,b).

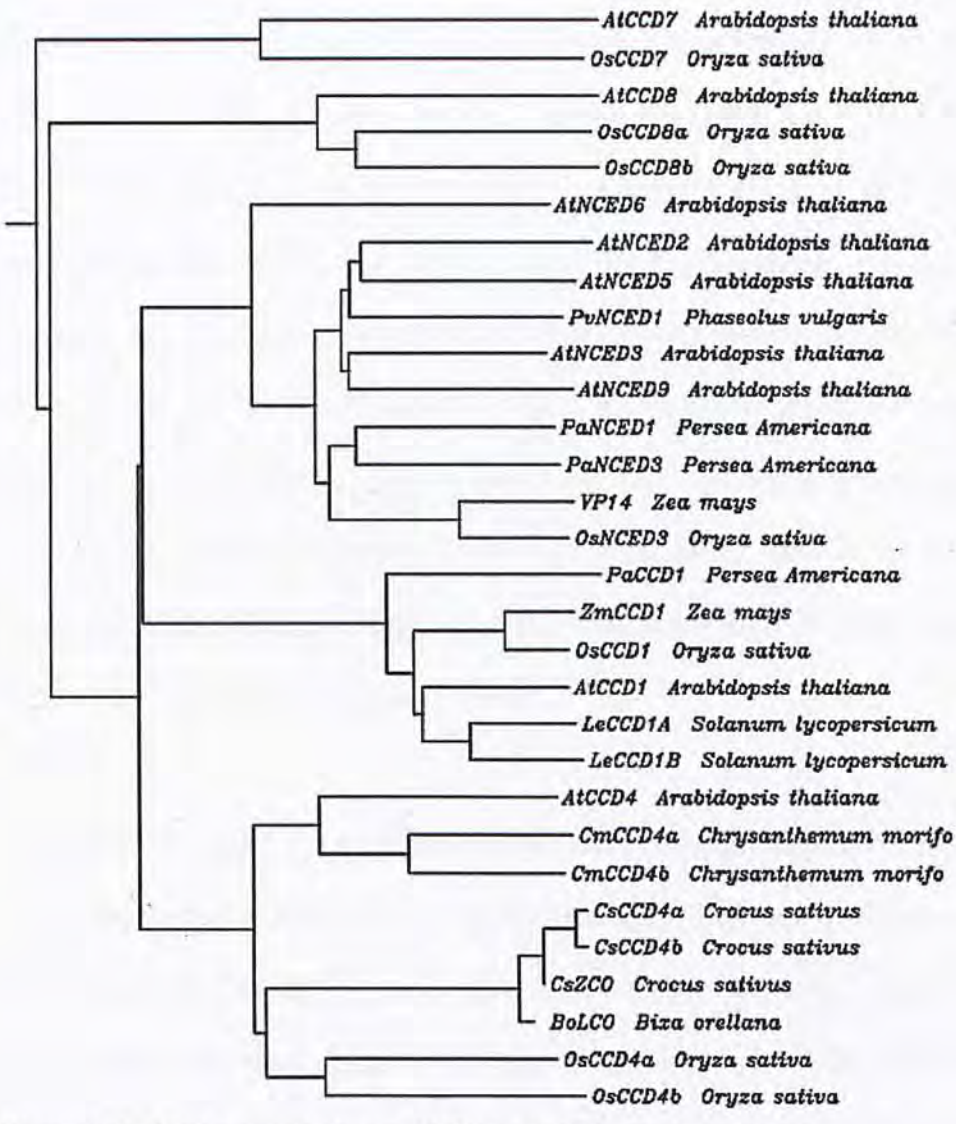

Figure 13. Phylogenetic tree of VP14 related genes in plants.

Abbreviatons: VP14 (GenBank accession number: NM_001112432) and ZmCCD1(DQ100346) of *Zea mays*; AtCCD1(NM_116217), AtNCED2(NM_117945), AtNCED3(NM_112304), AtCCD4(NM_118036), AtNCED5(NM_102749), AtNCED6(NM_113327), AtCCD7 (NM_1300 64), AtCCD8(NM_119434) and AtNCED9(NM_106486) of *Arabidopsis thaliana*; BoLCO (AJ489277), lycopene cleavage oxygenase of *Bixa orellana*; CmCCD4a (AB247158) , CmCCD4b (AB247160) of *Chrysanthemum morifolium*; CsCCD4a (EU523662), CsCCD4b(EU523663), CsZCO (zeaxanthin cleavage oxygenase; AJ489276) of *Crocus sativus* ; LeCCD1A (AY576001)and LeCCD1B (AY576002) of *Solanum lycopersicum*; OsCCD1(NM_001073927), OsCCD4a (NM_001054393), OsCCD4b (NM_001073218), OsCCD7(NM_001060026), OsCCD8a(NM_001049898), OsCCD8b (NM_001050764), OsNCED3(AY838899) of *Oryza sativa*; PaNCED1(AF224672), PaCCD1(AF224670) and PaNCED3 (AF224671) of *Persea Americana*; PvNCED1 (AF190462) of *Phaseolus vulgaris.*

To identify which *CCD*(s) is/ are responsible for carotenoid catabolism in rice seeds, their expression levels in seeds were analyzed by RT-PCR. Total RNA was extracted from wildtype whole rice seeds harvested at DAF 3, 6, 9, 12, 15, 18 and 21. To cover the entire seed developmental process, RNAs from different DAFs were pooled and used as one template for RT-PCR. Among the six *CCD*s, only *OsCCD1* was found highly expressed (figure 14A). To characterize *OsCCD1* expression at different developmental stages, RT-PCR was performed using RNA extracted from at different stages. Again, only *OsCCD1* showed high expression in all stages, whereas expression of other *CCD* members were absent throughout the development (figure 14B). This result indicates *OsCCD1* is the only *CCD* highly expressed in developing rice seeds.

To confirm the high level of expression and developmental regulation of *OsCCD1*, we qualified the mRNA levels independently by TaqMan real-time RT-PCR. Expression of *OsCCD1* was at the lowest level at early stages of seed development, and then started to increase from DAF3 to DAF6, followed by a gradual increment until the maximum at DAF9. A gradual reduction was observed as proceeds from DAF6 to DAF15, and then leveled off until full maturation (figure 15).

The *OsCCD1* expression pattern was consistent with the expression pattern of most carotenogenic genes in rice endosperm development (figure 12), especially *PSY* that encodes for the rate-limiting step in the carotenoid biosynthetic pathway. This result indicates that OsCCD1 may play a role in metabolizing carotenoids in developing rice endosperms.

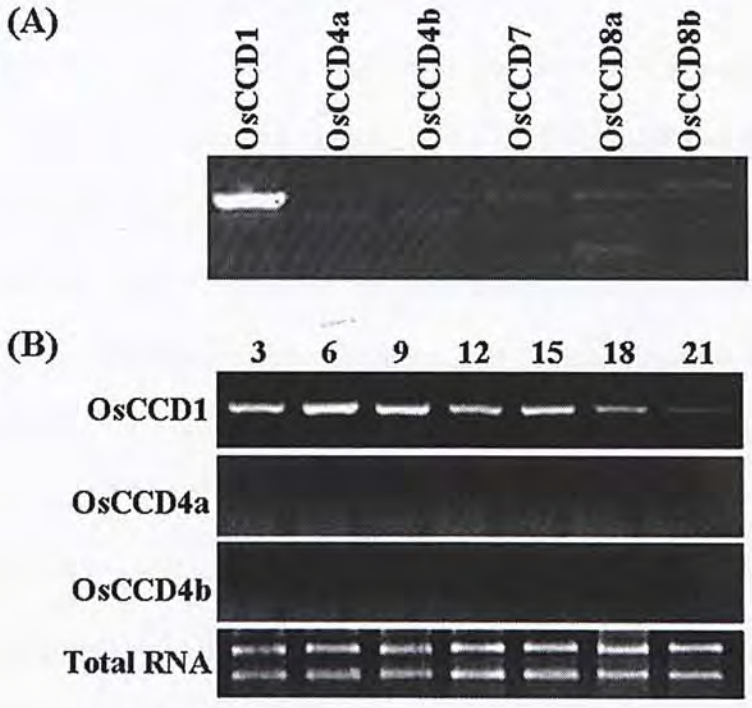

Figure 14. RT-PCR analyses of *CCD* family members in rice developing seeds. (A) RT-PCR analysis of *OsCCD1, 4a, 4b, 7, 8a* and *8b* on pooled total RNA of rice seeds at DAF3, 6, 9, 12, 15, 18 and 21. Weak bands were the results of non-specific amplification because their sizes did not match either the genomic DNA or cDNA. (B) RT-PCR analysis of *OsCCD1*, *4a*, *4b*, *7*, *8a* and *8b* in developing seeds. Only results of *OsCCD1, 4a* and *4b* were shown. Results of *OsCCD7*, *8a* and *8b* were similar to *OsCCD4.*

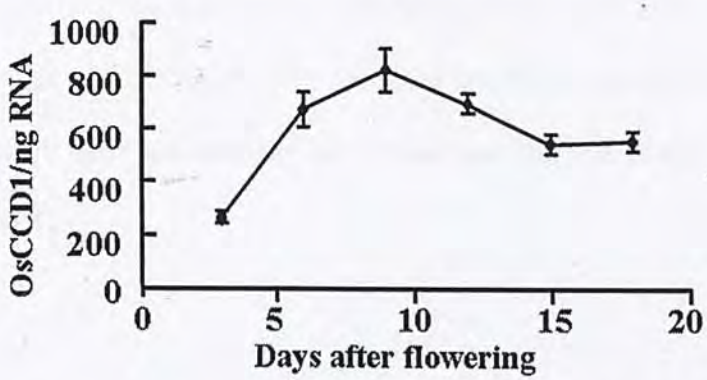

Figure 15. TaqMan real-time RT-PCR analysis of *OsCCD1 mRNA levels* in developing rice seeds.

5.3. Features of OsCCD1

To test whether *CCD1* orthologs in rice encodes a functional enzyme capable to cleave carotenoids, we cloned *OsCCD1* cDNA and analyzed its activity *in vivo*. Predicted *OsCCD1* cDNA was amplified by RT-PCR using high fidelity proof reading DNA polymerase *Pfx* (Invitrogen). cDNA sequence of *OsCCD1* was compared to the genomic sequence, and its gene structure was derived. It is shown that *OsCCD1* is 6835bp long, containing 14 exons and 13 introns (figure 16).

OsCCD1 protein was aligned with its orthologues using ClustalW2 programme. The predicted OsCCD1 is highly conserved with its apparent orthologues in one monocotyledonous, maize (*Zea mays*); and two dicotyledonous, tomato (*Solanum lycopersicum)* and avocado (*Persea Americana*) (figure 17). Their sequences were then analyzed by TargetP 1.1 programme (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al., 2000), the result showed that they contain four highly conserved His residues, and no plastid-targeting transit peptide was presented. These are the two characteristics of typical CCD1 proteins. From the alignment, OsCCD1 is 90% identical and 94% similar to ZmCCD1; 77% identical and 85% similar to AtCCD1; 77% identical and 86 % similar to LeCCD1A; 73% identical and 86 % similar to LeCCD1B; 73% identical and 81 % similar to PaCCD1. All the sequences are least conserved at the N-terminus, but highly conserved in other regions (figure 17).

Figure 16. Gene structure of *OsCCD1*. Boxes and lines denoted coding regions (exons) and introns, respectively.

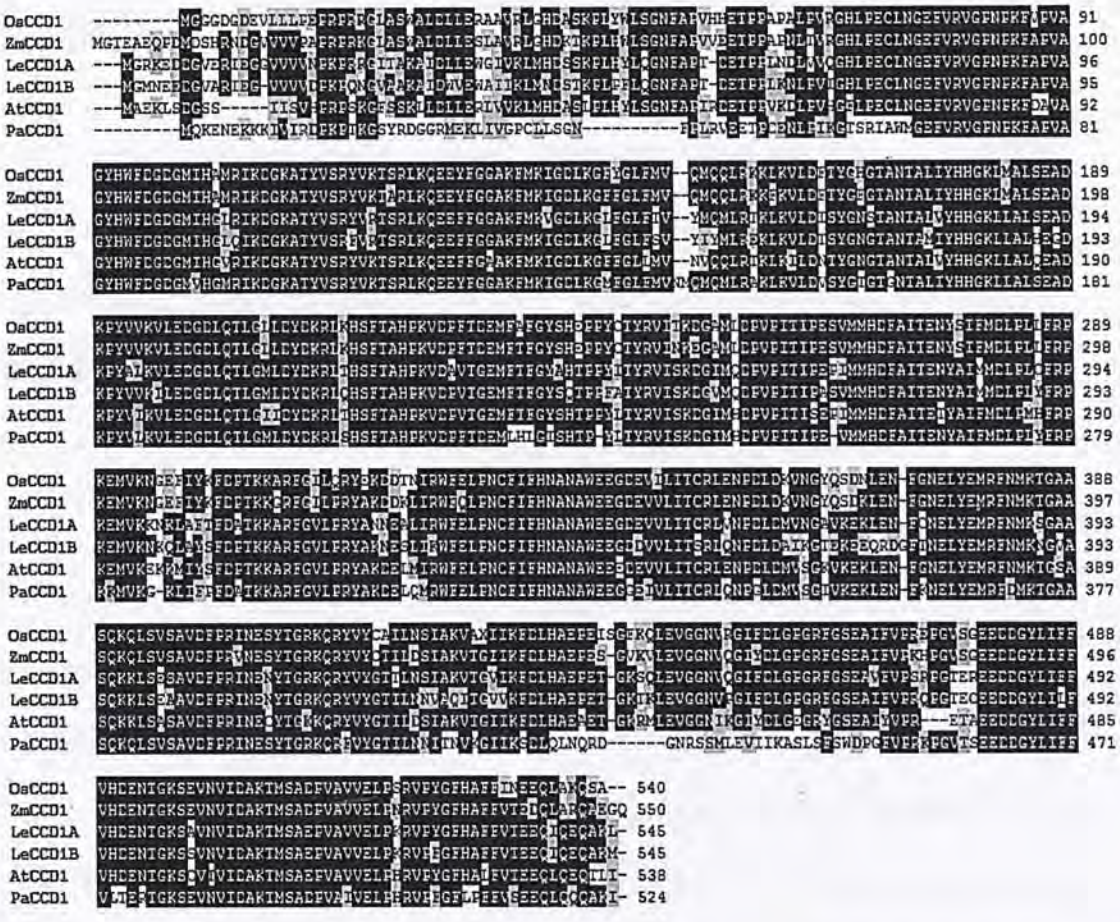

Figure 17. Alignment of OsCCD1 with related proteins using ClustalW2 program. Black and grey shadings represented conserved and highly similar residues, respectively. Abbreviations: OsCCD1 (NM_001073927); ZmCCD1(DQ100346) of *Zea mays*; LeCCD1A (AY576001)and LeCCD1B(AY576002) of *Solanum lycopersicum*; AtCCD1(NM_116217) of *Arabidopsis thaliana*; PaCCD1(AF224670) of *Persea Americana.*

To determine the enzymatic function of OsCCD1, we expressed OsCCD1 protein in *E.coli* cells. This *in vivo* assay in bacteria provides an easy method to detect enzymatic activity of CCDs as specific carotenoid levels can be detected visually by color changes or analyzed by gas chromatography and HPLC. Bacterial cells that were engineered to accumulate zeaxanthin, β-carotene and lycopene respectively displayed the color of each carotenoid. When these cells were induced to express OsCCD1, pigmentations in all three carotenoid-accumulating cells was either dramatically reduced or vanished (figure 18). HPLC analysis was performed to determine the amount of carotenoids extracted from cells with or without expression of OsCCD1. A 20 to 100 fold reduction of carotenoid content was observed in cell expressing OsCCD1 (data not shown). Thus, we conclude that OsCCD1 was enzymatically active. Its substrates include both linear (i.e. lycopene) and cyclic (i.e. zeaxanthin and β-carotene) carotenoids.

To investigate cleavage activities of OsCCD1, the emitted volatiles were collected by head-space method in the *E. coli* cells simultaneously synthesizing β-carotene and expressing OsCCD1. Volatiles were analyzed by gas chromatography. As indicated in figure 19, a new peak was detected at about 34.7minutes when OsCCD1 was expressed. Subsequently, analysis by gas chromatography-mass spectrometry (GC/MS) identified this peak is β-ionone. This indicates OsCCD1 cleaved on the carotenoid backbone at C9-C10 (C9'-C10') double bond, which is consistent with the activities of CCD1 in *Arabidopsis* (Schwartz et al., 2004) and tomato (Simkin et al., 2004a). Recently, additional cleavage activities of CCD1 were reported at C5-C6 (C5'-C6') and C7-C8 (C7'-C8') double bonds on lycopene and certain apolycopenals, respectively, under certain buffer conditions (Ilg et al., 2009; Vogel et al., 2008).

Figure 18. Expression of OsCCD1-GST fusion protein in *E.coli* cells that engineered to accumulate zeaxanthin, beta-carotene and lycopene. "-" without arabinose, "+" with arabinose.

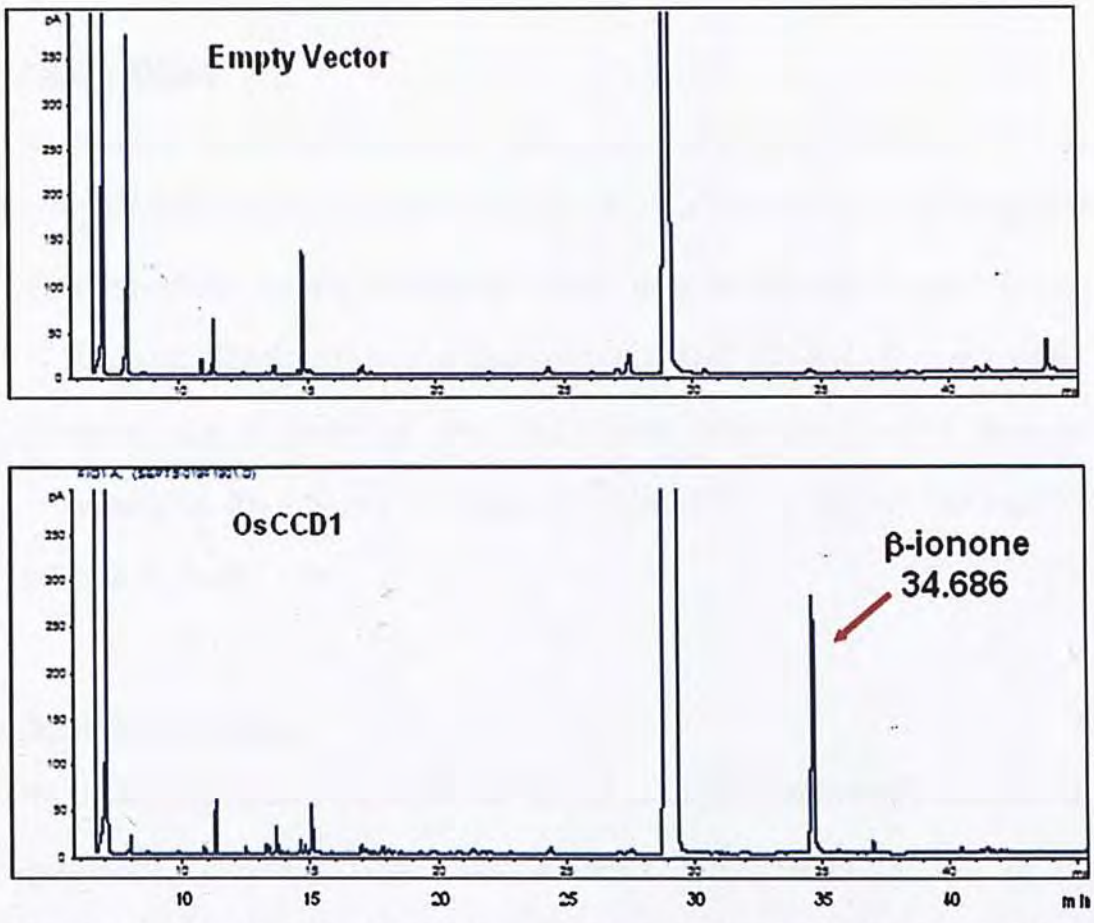

Figure 19. GC analysis of volatile products of beta-carotene accumulating *E.coli* cells that expressed OsCCD1. Empty vector was the negative control for the experiment.

5.4. Characterization of *OsCCD1*-knock down transgenic rice

To study the role of OsCCD1 in rice endosperm, RNA interference approach was applied. Two *OsCCD1* RNAi cassettes, "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1-RNAi" constructs (figure 4a and 4b), were built. *OsCCD1* RNAi in the first cassette is driven by of the maize constitutive ubiquitin promoter (*Ubi-1*), whereas the second one is driven by a rice endosperm-specific glutelin-C promoter (*GluC*). They were introduced into rice genome through *Agrobacteria*-mediated transformation. No visible alternation in color was observed in transformed calli for both constructs (data not shown).

GUS staining

Regenerated transgenic plants were further screened by GUS stain of root tissues using β-Glucuronidase Reporter Gene Staining Kit (Sigma). Only seedlings that showed positive staining results (figure 20A) were transplanted to field. 10 lines of "Ubi-OsCCD1-RNAi" and 30 lines of "GluC-OsCCD1-RNAi" were created. However, due to series of viral and fungus infections, only 6 lines of "Ubi-OsCCD1-RNAi" and 21 lines of "GluC-OsCCD1-RNAi" survived to produce T_1 seeds.

Southern blotting

To confirm transgene integration into the rice genome, Southern blot analysis was performed. Genomic DNA was extracted from leaves of T_0 or T_1 "Ubi-OsCCD1-RNAi" (5 lines) and "GluC-OsCCD1-RNAi" (14 lines) transgenic plants. After digestion with *Hin*dIII, the DNA blot was probed with a ^{32}P-labeled hygromycin B resistant gene. *Hin*dIII enzyme is chosen because the probe sequence does not contain this restriction site. As expected, no hybridized band

appeared in the wildtype plant (as negative control) (figure 21), indicating no cross-hybridization of the *Hyg* probe to rice DNA. In transgenic plants, one to three bands appeared in different lines (figure 21). This result indicated that there were one to three copies of transgene integrated into the genome. Differences in the fragment sizes indicated these lines were derived from independent transformation event.

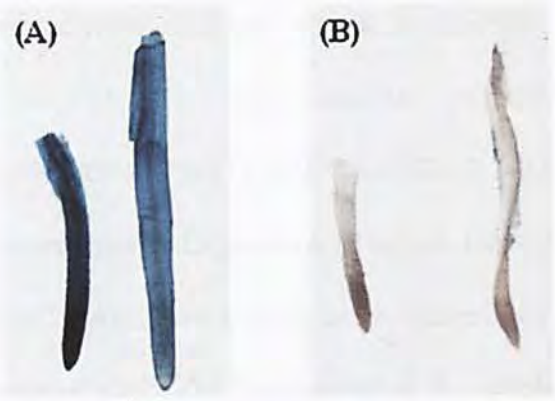

Figure 20. Identification of transgenic plants by GUS staining
(A) Positive result (B) Negative result

Figure 21. Southern blot analysis of "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1- RNAi" transgenic rice plants. Genomic DNA was extracted from each transgenic plant and digested with HindIII. The blot was hybridized with a ^{32}P-labeled Hyg resistance gene.

Expression of transgenes

Expression of *OsCCD1* in RNAi knockdown transgenic plants was checked by RT-PCR. RNA extracted from wildtype rice was used as the control for the normal expression level of *OsCCD1*. Gene specific primers, "OsCCD1-F3-RT" (5'-GTCGCTGGCTATCATTGGTT-3') and "OsCCD1-R2-RT" (5'-TATTGGCACA GGATCAAGCA-3') were used. Expression level of *OsCCD1* in all 4 lines of "Ubi-OsCCD1-RNAi" plants was significantly reduced as shown in figure 22, indicating the success of *OsCCD1* knockdown at the whole plant level. For the effect of *OsCCD1* knockdown in developing seeds, total RNA was extracted from whole seeds (developing seeds at stage 2 to 3, refer to figure 4.1). Result of RT-PCR was showed in figure 23. The expression level of *OsCCD1* in all 4 lines of "Ubi-OsCCD1-RNAi" plants was significantly decreased, whereas for "GluC-OsCCD1-RNAi", only line 9, 20 and 31 showed slight reductions in the expression.

There are several possible explanations for the results of "GluC-OsCCD1-RNAi" plants. Firstly, it may be due to the property of *GluC* promoter. As *GluC* promoter is endosperm-specific, expression of *OsCCD1* was not knocked-down in the seed coat and aleurone layer. So, it cannot be excluded that the RT-PCR signals were derived from the aleurone layer and seed coat, which made up relatively most of the developing seeds at the early stage of development. The second explanation would be about the expression profiles of *GluC* promoter. In the literature, analysis of *GluC* promoter expression only started as early as DAF7 (Qu et al. 2008), a time point where the lowest expression among all the stages was analyzed. No data was available for *GluC* activity at S2 (around DAF3) and S3 (around DAF5). So, we analyzed *GluC* expression in developing WT rice

endosperm by RT-PCR. Forward primer "Gt3-F1" (5′-TGGGTTCAGCCCAG ATTTAC-3′) and reverse primer "Gt3-R1" (5′-GGAGCAAAGACAGCCATCT C-3′) were used in the reaction. Result in figure 24 indicated *GluC* promoter expressed strongly in S3 whereas expressed extremely weak in S2. Having this property, when this promoter was used for building constructs, transgene driven by *GluC* promoter will only be expressed weakly in the earliest stage of development such as S2, so the expression of *OsCCD1* was not knockdown obviously. Another possibility is that some region of the gene may have poor efficiency in RNA interference such as in high GC regions (>50% GC).

Figure 22. RT-PCR analysis of *OsCCD1* expression in leaves of "Ubi-OsCCD1-RNAi" RNAi knockdown transgenic plants. Four transgenic lines (2, 4, 5 and 7) were analyzed.

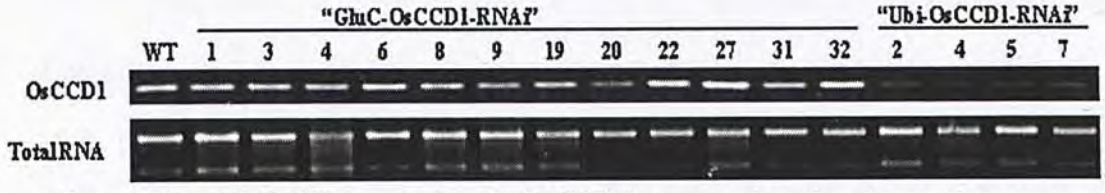

Figure 23. RT-PCR analysis of *OsCCD1* expression in the endosperm of OsCCD1-RNAi transgenic lines. Upper panel is the result of one-step RT-PCR. Lower panel is the normalization of RT-PCR using total RNA extracted from developing seeds at S2 to S3 (refer to the description in figure 11). Numbers on top are the line number of transgenic plants.

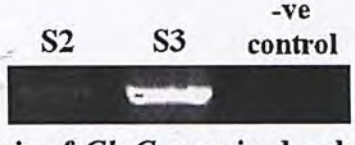

Figure 24. RT-PCR analysis of *GluC* gene in developing WT rice endosperm. Detailed descriptions of S2 and S3 was shown in figure 11.

5.5. Construction of "Super-Golden" rice

In the first part of our project, we knocked down the expression of *OsCCD1*, the only *CCD* responsible for carotenoid catabolism in rice seeds. Although accurate quantification has not been carried out, changes in seed pigmentation in *OsCCD1* knocked down lines were visible, strongly indicating the accumulation of carotenoids. This result demonstrated that, if confirmed by HPLC analysis, blocking OsCCD1-mediated carotenoid catabolism resulted in increased carotenoid accumulation. These results also indicate that, consistent with our RT-PCR analysis on genes of the carotenoid biosynthetic pathway, carotenoids are synthesized in rice seeds although at low level because of low *PSY* expression. The limited amount of carotenoids is further subjected to "degradation" by OsCCD1, resulting in an extremely low accumulation of carotenoids in rice seeds. This raises an important question as to how much a role OsCCD1 played in Golden rice (Paine et al., 2005; Ye et al., 2000) where substrates were at high levels. To address this question, we attempted to create transgenic rice by increasing carotenoid biosynthesis in conjunction with reducing carotenoid catabolism.

Two transgenic constructs namely "pGT-PCC" and "pGYGC" were designed (figure 6), in which "pGT-PCC" was successfully built and introduced into rice genome by *Agrobacteria*-mediated transformation, whereas "pGYGC" is still in the process of construction. "pGT-PCC" aims to over-express *CRTI/ZmPSY1* and to RNAi knock down *OsCCD1* at the same time in endosperm-specific manner. "pGYGC" acts as an control for "pGT-PCC" by only over-expressing *CRTI/ZmPSY1* in the endosperm without knocking down *OsCCD1*. As discussed previously, different from the golden rice created in 2000 and 2005, a stronger

endosperm-specific promoter, *GluC* promoter is used for driving the expression of our transgenes. Besides, the *crtI* was cloned from *Pantoea agglomeran* (previously classified as *Erwinia herbicola*; Acc: M38423) instead of *Pantoea ananatis* (previously classified as classified as *Erwinia uredovora;* Acc: D90087).

5.6. Phenotypic characterization of PCC transgenic rice

"pGT-PCC" construct was transformed into rice callus by *Agrobacteria*-mediated transformation. After transformation, callus turned from light yellow into bright yellow, and some portions of the callus even became orange (figure 25), indicating carotenoid biosynthetic pathway is functional in transgenic cells although the promoter used was not designed to express in vegetative cells. However, information about glutelin promoter specificity in callus cells is lacking. The yellow color of callus indicated 1) the engineered carotenoid biosynthetic pathway is functional; 2) *Gt1* and *GluC* promoters may have leaky expression in rice callus.

The "pGT-PCC" transgenic rice lines were closely monitored for phenotypes that might be associated with the transformation. No abnormal phenotypes were detected in plant growth, development and seed morphology. Instead, T_1 "pGT-PCC" transgenic seeds were dramatically golden-orange in color. As shown in figure 26, the pigmentation is far more intense than the "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1-RNAi" transgenic seeds. We are in the process of quantifying the content of carotenoids in the "pGT-PCC" seeds before a comparison with reference to previous Golden rice can be made. The strong accumulation of carotenoids in the PCC transgenic seeds may exceed the previous Golden rice considering the attenuation of OsCCD1 activity as indicated in

reduced carotenoid degradation in WT rice seeds. Precise genetic analysis with or without *OsCCD1* expression will be conducted in future research.

5.7. HPLC analysis on seed carotenoid content

Due to viral and fungus infection, T_1 seeds of "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1-RNAi" produced were not enough for HPLC analysis. To produce more seeds, 4 lines from each of the constructs were randomly selected to grow for another season. T_2 seeds obtained will be subjected to detail analysis on their carotenoid profiles in future research.

Figure 25. Callus color before and after transformation with "pGT-PCC" construct. "Before", callus induced from mature rice seeds, on solid 2N6 medium. "After", rice callus transformed by construct "pGT-PCC", on solid 2N6-TCH selection medium containing hygromycin.

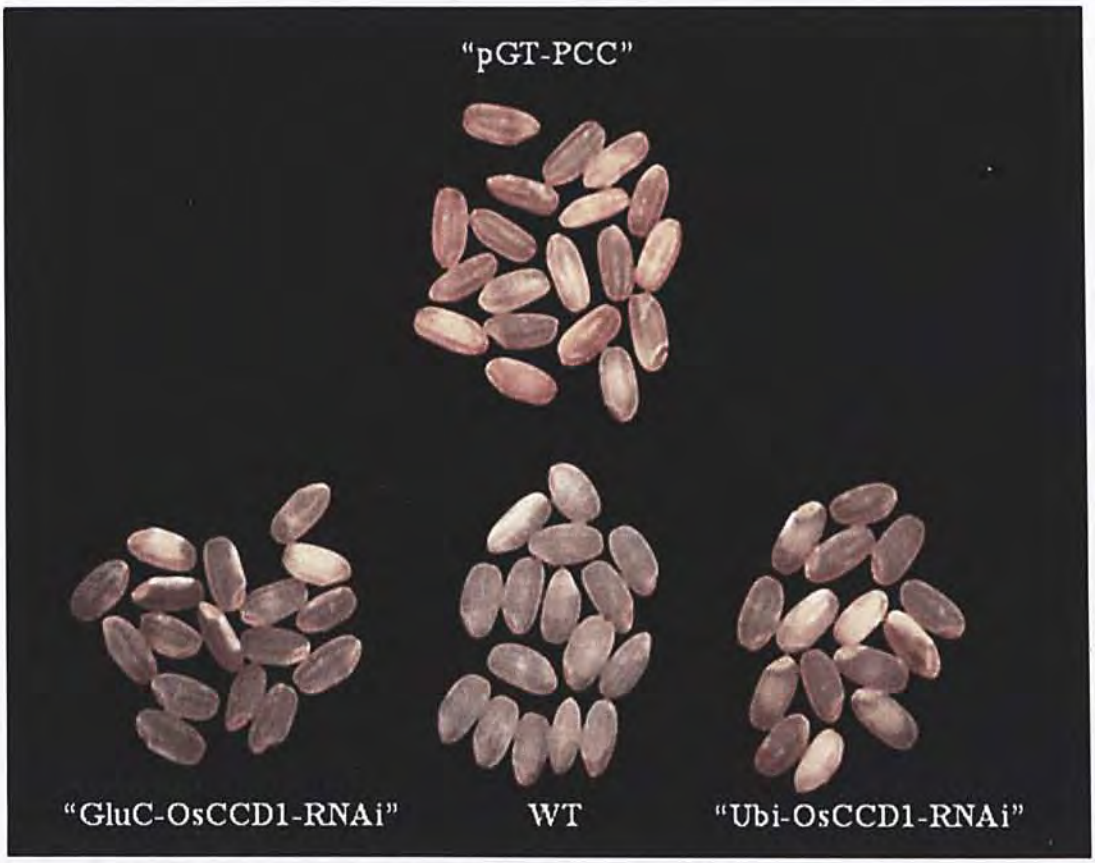

Figure 26. Comparison of "pGT-PCC", "Ubi-OsCCD1-RNAi" and "GluC-OsCCD1 -RNAi" transgenic seeds with wildtype seeds (WT).

Chapter 6. Discussion

Active carotenoid biosynthesis exists in the developing rice embryo and endosperm

Due to the low expression or low activity of phytoene synthase and one or more genes in the desaturation/isomerization system (system composed of four enzymes including PDS, ZDS, Z-ISO and CRTISO; refer to figure 1), wild type rice seeds are white and apparently lack of carotenoids (Burkhardt et al., 1997; Schaub et al., 2005). To boost up carotenoid biosynthesis in rice, bacterial phytoene desaturase (*CrtI*) and phytoene synthase (*Psy*) from daffodil or maize have been introduced into rice genome, leading to the creation of golden rice with enhanced carotenoid biosynthesis (Paine et al., 2005; Ye et al., 2000). In our study, we attempt to further increase carotenoid accumulation by minimizing its catabolism, in conjunction with enhanced biosynthesis. .

Before addressing catabolism, we must first prove the existence of carotenoid biosynthesis in rice seeds. In a study about rice *lcyβ* mutants, pink embryo was observed indicating accumulation of lycopene (Fang et al., 2008). This accumulation of intermediate metabolite indicates carotenogenic pathway is active as least in rice embryo. In our study, we found that all the carotenogenic genes are expressed in the developing rice endosperm albeit in different levels (figure 12). Although no visually pink color was observed in the endosperm of *lcyβ* mutant, it cannot exclude the possibility that carotenoids were accumulated in small amount that exceeds the limit of visual detection. It would be interesting to know the carotenoid level in *lcyβ* mutant endosperm.

If carotenoids are synthesized in rice endosperm, due to its hydrophobic nature, it will be stored in the amyloplast membrane. In potato, carotenoids mainly xanthophylls like lutein are found in membrane of amyloplast, in which 17-22% of them are in esterified forms (Breithaupt and Bamedi, 2002; Fishwick and Wright, 1980). In maize, barley, oat and even the nearly white wheat seeds, lutein is the major carotenoid presented whereas β-carotene is presented in minority (Janick-Buckner et al., 1999; Konopka et al., 2004; Panfili et al., 2004; Zandomeneghi et al., 2000). All these suggest that seed endosperm is capable to store carotenoids especially lutein.

CCD1 and carotenoid catabolism in plant seeds

In our study, we identified OsCCD1 as the only candidate for carotenoid catabolism in rice seeds. Linkage between CCD1 and carotenoid catabolism in seeds were previously documented in maize and *Arabidopsis*. *ZmCCD1* encoded by *White cap locus (Wc)* is found to be the causative gene for carotenoid breakdown in endosperm in dosage-dependent manner (Tan et al., unpublished data; Timmermans et al., 2004). Further evidence comes from a transformation experiment (Aluru et al., 2008). *Wc* containing maize Hi-II (white kernel maize) was transformed by carotenogenic genes *CrtB* and *CrtI*. Although the carotenogenic pathway was reconstructed through transgenic event, no extra carotenoids were accumulated in the endosperm when the trasngenes were driven by a regular endosperm-specific promoter. Significant amount of carotenoids can only be accumulated when stronger promoter was used. This is a piece of indirect evidence suggesting that ZmCCD1 in Hi-II maize endosperm was very active thus hindered carotenoid accumulation. One explanation is that only when the amount of carotenoid synthesized surpassing certain amount, *Wc* was saturated and thus

allowed the accumulation of carotenoids for detection.

Consistent with the finding in maize, when CCD1 orthologue in *Arabidopsis* was knocked out by T-DNA insertion, a 37% increment in total carotenoid content was detected in seeds without any abnormal phenotype (Auldridge et al., 2006). This allows us to have a brief understanding of CCD1's role in carotenoid catabolism. However, as *Arabidopsis* is dicotyledonous whereas rice is monocotyledonous, structures of their seeds are different. Mature *Arabidopsis* seed consists mainly of embryo, which is surrounded by a single-cell layer of endosperm. In comparison, rice seed has typical structure of a caryopsis, which consists of a large starchy endosperm and an embryo. Since the mechanism of carotenoid metabolism in rice and *Arabidopsis* may not be the same, it is worthwhile to further study CCD1 function in monocots. From our result, when *OsCCD1* was knocked down in wildtype rice background, transgenic rice seeds were visually pale yellow in color instead of white (figure 26). Although HPLC analysis has not yet been performed, with reference to OsCCD1 activities in carotenoid accumulating *E.coli*, the yellow pigment is highly possible to be carotenoids or apocarotneoids. If it is so, this would demonstrate that both active carotenoid biosynthesis and catabolism exist in rice seeds, although the biosynthesis is low.

Biological function of CCD1 in plant

Members of CCD1 subfamily are highly conserved in plant kingdom (figure 13 and 17). This indicates CCD1s may have important function(s), and thus there is high selection pressure causing their low divergence. They distinguish themselves from other CCD members by the absence of plastid-localizing signal. It is the only CCD localized in the cytosol instead of plastids (Auldridge et al., 2006).

Differences in localizations reflect CCD1 may exert functions that are very different from the other CCDs.

In our project, we study CCD1's possible involvement in growth and development. We created OsCCD1 RNAi transgenic plants that were driven by constitutive *Ubi* promoter. All the transgenic plants looked like normal wildtype plants (data not shown). No abnormal phenotypes such as viviparous seeds or delayed germination were observed (data not shown). Seed dormancy is regulated by balance between gibberellins (GA) and abscisic acid (ABA). Gibberellins (GA) is synthesized through terpenoid pathway and can promote germination. For abscisic acid (ABA), it is derived from carotenoids violaxanthin and neoxanthin. It can maintain seed dormancy and inhibit seed germination. In tobacco and *Arabidopsis*, when carotenoid biosynthesis is enhanced, increased ABA accumulation is accompanied with delayed germination (Frey et al., 1999; Lindgren et al., 2003). If CCD1 cleaved the upstream carotenoids in the biosynthetic pathway, knocking down *OsCCD1* would lead to more substrate available for ABA biosynthesis, and thus more ABA would be produced and lead to delayed germination. However, as no such phenotype was observed in our transgenic plants, this suggests that degradation of carotenoids by OsCCD1 in vegetative tissue is limited or highly regulated. Since pre-harvest sprouting has not been observed in "Ubi-OsCCD1-RNAi" transgenic plants (data not shown), OsCCD1in vegetative tissues plant may not be able to cleave zeaxanthin, violaxanthin and their upstream carotenoids. For the second possibility, cytosolic OsCCD1 (Auldridge et al., 2006) cannot reach its carotenoid substrates which are synthesized and located in plastids. If it is the first case, we may have to reconsider substrate choice for CCD1 enzyme in plant.

Bacterial *in vivo* studies suggested substrates of CCD1 are various C40 carotenoids. As carotenoids are localized in plastids, the way of how cytosolic CCD1 reaches its substrates is still a puzzle. Three hypotheses have been proposed (Walter et al., 2010). The first hypothesis suggests that substrates of CCD1 in plant are C27 apocarotenoids instead of C40 carotenoids, and C40 carotenoids are cleaved sequentially by different CCDs in different compartments (Floss and Walter, 2009). Supporting evidences have been demonstrated through transgenic approaches. When *CCD1* expression was knocked down in mycorrhizal roots of maize and *Medicago trucncatula* by RNAi, there were reductions in C13 and C14 apocarotenoids accumulations. On the contrary, C27 apocarotenoids accumulation was increased (Floss et al., 2008; Sun et al., 2008). When OsCCD1 was overexpressed in the endosperm of golden rice, although no conclusive data in the seed total carotenoid content, *in vitro* incubation assay showed there were cleavage activities on C27 apocarotenoids but not on C40 carotenoids (Ilg et al., 2010). All these studies provided new views on the substrate choices of CCD1 in plants (Floss and Walter, 2009).

CCD1 may work downstream of other CCD family members such as CCD4 and CCD7/CCD8 (Floss and Walter, 2009). From biochemical analysis, substrate of CCD4 and CCD7 are proposed to be C40 carotenoids whereas substrates CCD8 is proposed to be the C27 apocarotenoids produced by CCD7 cleavage activity (Huang et al., 2009; Schwartz et al., 2004). As CCD7 and CCD8 function in strigolactone biosynthetic pathway sequentially, if CCD1 functions downstream of CCD7, knocking down CCD1 should provide more substrate for CCD8 and thus more strigolactone would be synthesized. However, in our experiments, there was no reduction in branching observed in our "Ubi-OsCCD1-RNAi" transgenic

plants. This suggests that there was no significant increment of branching inhibiting strigolactone being synthesized. In fact, some of the T_1 trasngenic plants even showed frequent generation of tertiary tillers and large branching angles at nodes. As these phenomenons were observed also in some "GluC-OsCCD1-RNAi" transgenic plants, these may be caused by field conditions instead of the effect of *OsCCD1* knockdown. So, it would be too early to draw any conclusion from the phenotypes of these transgenic plants.

Another hypothesis proposed that when the cell is damaged or plastids undergo degeneration, C40 carotenoids that are localized on plastid membrane would be released into the cytosol (Walter et al., 2010). This would allow cytosolic CCD1 gain access and perform cleavage activities on the substrates, leading to the production of aromatic volatiles. These volatiles may somehow contribute to the plant defense mechanisms against damages by herbivores or insects. Until now, no experiment has been reported to prove this hypothesis.

Based on homology search, an orthologue of CCD1, NosCCD (previously named as NSC1), has been identified in cyanobacteria *Nostoc* sp. PCC7120 (Marasco et al., 2006). It shared two important features with plant CCD1, which are the cytosolic localizations, as well as the abilities to cleave various C40 carotenoids and apocarotenoids at multiple double bonds such as C9-C10 (C9'-C10') bond (Marasco et al., 2006; Scherzinger and Al-Babili, 2008). As cytosolic NosCCD can be induced by high-light, it is proposed to be the scavenger of cytosolic apocarotenoids produced under high-light stress (Scherzinger and Al-Babili, 2008). With reference to NosCCD, it is speculated that CCD1 may exert a similar function in plant and generate aromatic volatiles as products of its reaction (Ilg et

al., 2010). Although this may explain the broad spectrum of CCD1 substrates and cleavage activities, more experiments are required before drawing any conclusion on this hypothesis. CCD1 function in plant is still a mystery.

To sum up, we did not observe any visible effect on knocking down *OsCCD1* in whole plant level. However, in seeds, clear alternation to yellow-orange color was observed, indicating the accumulation of pigmented carotenoids and/ or apocarotenoids in seed endosperm. This suggests that CCD1 may have important role in carotenoid accumulation in rice seeds.

To further explore CCD1 substrates in plant, HPLC will be done to analyze carotenoid and apocarotenoids contents in transgenic seeds. To observe the changes more obviously, *OsCCD1* knockdown effect will be monitored in modified Golden rice background in future studies.

Chapter 7. Conclusion

There is active carotenoid biosynthesis in developing rice seed albeit at low level, and OsCCD1 is the only member in CCD family most likely to be responsible for carotenoid turnover. Knocking down *OsCCD1*expression turned the seeds from white into yellow indicated increment of yellow-pigmented component accumulations, which is highly possible to be pro-vitamin A carotenoids. There is no other abnormal alternation of growth and development observed in transgenic plants.

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